SEXUAL HORMONES IN ACHLYA. VII. THE HORMONAL MECHANISM IN HOMOTHALLIC SPECIES[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 614

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Introduction

The sexual reproductive process in two heterothallic species of *Achlya*, a genus of the ubiquitous "water molds," has been shown to be initiated and coordinated throughout its entire course by a series of specific, diffusible hormones (**7, 8, 10, 11, 12, 14, 15**). The over-all picture of the hormonal mechanism has recently been revised to include the results of continuing work, primarily in respect to two heretofore undescribed hormones operative in the initiation of the sexual reaction (**14**). The mechanism, as now understood, comprises seven distinct hormonal activities, four secreted by the ♂ and three secreted by the ♀. A detailed description of the roles of the several successive hormones has been published elsewhere and will not be included here. A diagrammatic summary of the mechanism, however, is required as a point of reference and is included in the body of the paper (fig. 11).

The morphological and sequential aspects of the sexual processes of homothallic (☿) species of *Achlya* and related genera correspond almost exactly with those of the process in *A. bisexualis* and *A. ambisexualis*, the two heterothallic species in which the physiological basis of sexual reproduction has been intensively studied. Since the production, development, and proper function of the sexual organs, as well as the invariable sequence of the several stages of the sexual reaction, result from the activities of specific hormones in heterothallic species, a comparable mechanism is indicated for homothallic forms. Further indicative evidence for such a correlative mechanism in homothallic species is seen in the definite, although usually weak and abortive, interspecific reactions between homothallic and heterothallic plants. This effect, first described by COUCH (**4**) in a mating of *Thraustotheca primoachlya* and *Dictyuchus monosporus* ♀, has been repeatedly observed by the author in numerous interspecific and intergeneric matings involving homothallic plants as one or both members of the mating pair. Finally, the phenomenon of redifferentiation of sexual organs in homothallic water molds described by HUMPHREY (**5**), MAURIZIO (**6**), and COKER (**3**)—i.e., antheridial hyphae arising from oogonial initials or the final differentiation of an oogonium in the tip of an antheridial hypha—suggests a hormonal mechanism in which quantitative changes of the hormonal balance result in these seemingly anomalous reversals.

The first concrete evidence of the presence of a hormonal mechanism in homothallic species came by accident in the spring of 1946. An intense search was being made in southern Wisconsin and northern Illinois for a pure-♂ strain of *A. ambisexualis*, and any isolate belonging to the genus *Achlya* which appeared

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to be self-sterile was hopefully, if prematurely, tested with hormone A of *A. bisexualis* ♀. A number of isolates which were self-sterile at 3 days of age showed positive responses to hormone A—i.e., produced antheridial hyphae—but initiated oogonial production a day later. These particular isolates were later identified as *A. apiculata;* further tests with numerous other homothallic isolates showed that the effect was not restricted to this species. From these preliminary observations it was obvious that different species reacted differently, both qualitatively and quantitatively, to the hormone of *A. bisexualis* ♀ which induces antheridial hyphae. Of a number of homothallic isolates tested with hormone A, one of *A. americana*, collected at Lake Geneva, Wisconsin, reacted most vigorously and most uniformly. The pattern of the reaction in this species also corresponded most closely with that of the ♂ of *A. ambisexualis*, the plant previously used in the hormone study and in which antheridial hyphae are uniformly produced along the entire lengths of the vegetative hyphae. This isolate of *A. americana*, the same one used in the present study, was employed for the quantitative assay of hormone A during a period of more than a year (1) until a pure-♂ strain of *A. ambisexualis* was obtained late in 1947.

The preliminary observations described above indicated parallel or even identical stages in the mechanism of initiation of the sexual reaction in homothallic and heterothallic species and pointed out the possibility that each homothallic species possesses a hormonal mechanism comparable with the combined chemical activities of the ♂ *and* ♀ strains of heterothallic forms. It is the purpose of the current paper to present the results of a study undertaken to elucidate the mechanism of sexual reproductive processes in homothallic water molds. The objectives were two: (*a*) to determine the degree of similarity or dissimilarity between the homothallic pattern of sexual reaction and the combined ♂ and ♀ pattern in heterothallic species and (*b*) to determine the occurrence of sexual hormonal mechanisms in various homothallic members of *Achlya* and of other genera of the Saprolegniaceae. Each of these objectives has been only partially achieved, but it is believed that the findings to date constitute a significant contribution to our knowledge of sexual processes in the aquatic fungi.

Material and methods

The biological materials and the experimental procedures used closely correspond with those employed in previous studies on sexual hormones in *Achlya*. The actual isolates of the heterothallic species are entirely new, but all major results of studies made prior to 1942 have been confirmed with them. The following brief description, therefore, serves chiefly to indicate the extensions of, or departures from, previously described materials and experimental procedures.

The plant materials comprise two ♂ and two ♀ strains of heterothallic species of *Achlya*, twelve isolates of homothallic species of *Achlya* of which only three have been identified, and two homothallic species of *Thraustotheca*. The identity and source of each of these plant materials are given in table 1.

Three hormone concentrates derived from heterothallic strains and described more fully elsewhere (14) have been used in the work. These comprise three members of the hormone A-complex: (*a*) hormone A, from *A. bisexualis* ♀, in acetone solution and having a titer of 5×10^5 units/ml; (*b*) hormone A^1 (previously

designated as A′ [12]), from *A. ambisexualis* ♂; and (*c*) hormone A^2, from *A. bisexualis* ♀.

The filtrates from three homothallic species, *A. americana*, *Thraustotheca clavata*, and *T. primoachlya*, have been fractionated into acetone-soluble and water-soluble (acetone-insoluble) fractions and tested as described below. Assuming identical mechanisms in homothallic and heterothallic forms, the acetone-soluble fraction should contain hormone A; the water-soluble fraction, hormones A^1 and A^2 (14).

The filtrates of all plants were obtained from aerated mass cultures in which 5 gm. of hemp seed per liter served as substrate (15).

Vegetative cultures have been maintained in a sterile condition on a medium consisting of 3 gm. soluble starch, 1 gm. peptone, and 20 gm. agar per liter of water distilled from Pyrex glass and containing 10^{-4} M KH_2PO_4 and 1 ml/liter of Hoagland's A-Z solution. A medium differing from this only by the addition of 1 gm. of inositol per liter has been used whenever matings have been made on a semisolid substrate.

Quantitative tests for the hormones of the A-complex were made as previously described (11) with ♂ plants of *A. ambisexualis* grown to the age of 4 days in water on halved hemp seed. *A. americana* and other homothallic species used in the comparative studies of the responses to the hormones of the A-complex were tested when 3 days old. Even at this age some sexual activity had been spontaneously initiated prior to the tests but not in such intensity as to obscure the results. All counts of antheridial hyphae were made 3–4 hours after the introduction of the plants into the test solutions.

A most useful technique for the mating of plants on opposite sides of a permeable membrane, either for the purposes of a specific study as that described herein or for a more generalized demonstration of hormonal activities in these aquatic

TABLE 1

IDENTITY AND SOURCE OF PLANT MATERIALS

Isolate no.	Species	Sexual strain	Source	Collector	Date
734	*A. ambisexualis*	♀	Lake Calumet, Chicago	JRR	Aug., 1948
E87	*A. ambisexualis*	♂	Cambridge, Eng.	JRR	Oct., 1947
355	*A. bisexualis*	♀	Joliet, Ill.	EDWARD BOAS	July, 1946
E247	*A. bisexualis**	♂	Lake Windermere, Eng.	JRR	Oct., 1947
6	*A. americana*	☿	Lake Geneva, Wis.	JRR	Apr., 1946
47, etc.	*A. apiculata*†	☿	Genoa City, Wis.	JRR	Apr., 1946
—	*A. recurva*	☿	Chapel Hill, N.C.	A. W. ZIEGLER	June, 1947
1, 2, 51, 86, 109, 120, 187	*A. spp.*	☿	Southern Wisconsin and northern Illinois	JRR	Apr., —— June, 1946
—	*A. sp.*	☿	Cambridge, Mass.	R. M. PAGE	Aug., 1947
—	*T. primoachlya*	☿	———, Ill.	E. BENECKE	——, 1948
—	*T. clavata*	☿	Chapel Hill, N.C.	J. N. COUCH	Jan., 1950
E235	*T. clavata*‡	☿	Lake Windermere, Eng.	JRR	Oct., 1947

*The identity of E247 is not certain. Strong mutual mating responses occur between it and *A. bisexualis* ♀, but most of the resulting oospores abort. The isolate is probably a pure-♂ strain of an undescribed heterothallic species.

† *A. apiculata* grew poorly under the cultural conditions routinely employed for saprolegniaceous fungi. Several isolations were made, but all were lost within a few months after their collection and were available only for the preliminary observations.

‡ Lost in 1949.

fungi, has been perfected during the course of the investigation. A very thin layer of agar medium, 1 mm. or less in depth, is poured into a Petri dish and allowed to solidify. A small circular hole is then made in this layer of medium on one side of the plate with a metal tube, sharpened at the end and sterilized by flaming. An inoculum, consisting of a small cube of agar cut from the periphery of a vigorously growing mycelium, is then transferred to the hole. A disk of sterile dialyzing "Cellphane," cut to extend to the edge of the dish, is then laid on the surface of the agar, and any bubbles of air trapped beneath the membrane are worked out by means of a small, bent glass rod. The membranes may be easily handled if they are stacked alternately with pieces of filter paper of the same size, wetted thoroughly, drained of excess water, and sterilized by autoclaving. When a filter paper is picked up from the stack with forceps, the adjacent membrane adheres to it, and both are transferred to the plate; the filter paper is then easily peeled off and removed. Additional agar, cooled to a temperature of about 40° C., is then poured over the membrane to a depth of about 1 mm. and, after solidifying, is inoculated on its surface on the opposite side of the plate from the inoculum in the layer of medium beneath. The growth rate of the mycelium on the lower layer of medium is approximately normal, and the two mycelia overlap in 3–4 days. Strict care is necessary to prevent contamination, since the plate must be opened a minimum of seven times in this procedure.

Experimentation and results

Three distinct lines of experimentation have yielded pertinent and corroborative information on the mechanism of sexual reproduction in homothallic plants. These are (*a*) matings of homothallic × ♂ and ♀ strains of heterothallic species in water and in agar; (*b*) matings of homothallic × heterothallic species in an agar medium in which the reacting plants are physically separated by interposed permeable membranes; and (*c*) induction of sexual activities in homothallic and heterothallic species by means of filtrates from plants of the contrasting sexual type.

Matings in water

Matings in water have been made between three homothallic species, *A. americana*, *T. clavata*, and *T. primoachlya*, and all four of the tester strains of the two heterothallic species listed in table 1. The results of these matings were as follows:

A. americana × *A. ambisexualis* ♂: No discernible response was elicited in either of the two plants.

——— × *A. ambisexualis* ♀: A fairly intense production of antheridial hyphae on the ☿ was followed by the production of relatively few oogonial initials on the ♀ (fig. 1, *1*). Subsequent stages of the reaction appeared to be quite normal. Normal oogonia, producing matured oospores, were formed on the ☿ mate on all portions of the mycelium except in the region of intermingling with the ♀ mycelium.

——— × *A. bisexualis* ♂: No reaction in either mate.

——— × *A. bisexualis* ♀: A very intense production of antheridial hyphae was induced on the ☿, and simultaneously all previously formed oogonial initials not yet delimited by basal septa either redifferentiated or degenerated (see below for the behavior of ☿ species in high concentrations of hormone A). No response was induced in the ♀.

T. clavata × *A. ambisexualis* ♂: Slight

production of antheridial hyphae was induced on the ♂ in those portions of its mycelium contiguous with the ♀ (fig. 1, 2). No discernible reaction was induced in the ☿.

A different isolate of *T. clavata*, E235, collected in England in 1947 and maintained in culture for 2 years, induced a much more vigorous reaction in *A. ambisexualis* ♂ than did the isolate from North Carolina. Antheridial hyphae from the ♂ plant were attracted in this mating to the oogonial initials of the ☿ in such numbers as apparently to prevent, in the case of many oogonia, the normal function of the ♂ sexual organs of the ☿ plant. Oospores in oogonia which appeared to be reached only by antheridia from the ♂ plant developed normally. The differences in the reactions of these two isolates of *T. clavata* may have significance as indicated in the discussion. Unfortunately, the English strain was lost, and a direct comparison of the behavior of the two was not possible.

——— × *A. ambisexualis* ♀: The reaction in this mating was intense and appeared to be perfectly normal throughout the entire series of mating responses (fig. 1, 3–5). Antheridial hyphae were produced in abundance on the ☿ in the region of intermingling of the two mycelia, and numerous oogonial initials were formed on the ♀ plant, chiefly in the region of intermingling but scattered sparsely over the entire mycelium. Eccentric oospores (fig. 1, 5) were matured in the oogonia in approximately the percentage characteristic for mating of ♂ × ♀ strains of *A. ambisexualis* under laboratory conditions. Quantitatively the reaction (fig. 1, 4) was comparable in intensity with that between strong-♂ and strong-♀ strains of *A. ambisexualis*. The normal hermaphroditic sexual proc-

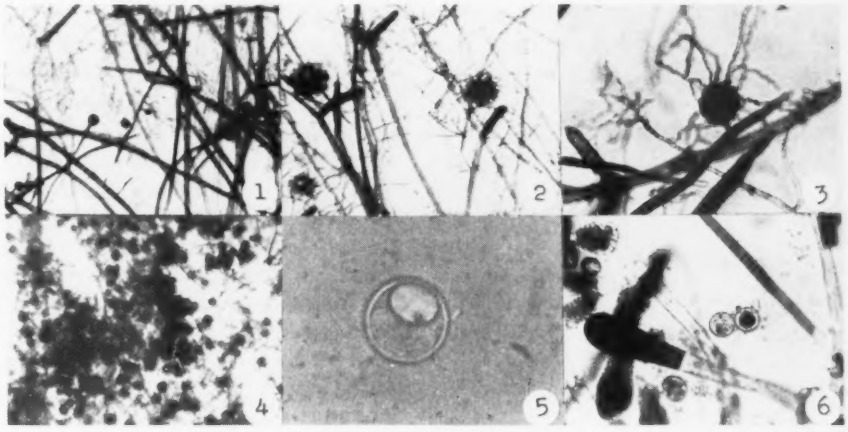

FIG. 1.—Reactions between homothallic and heterothallic species mated in water. (*1*) *A. americana* × *A. ambisexualis* ♀; antheridial hyphal and oogonial production on ☿ and ♀ mycelia, respectively. (*2*) *T. clavata* × *A. ambisexualis* ♂; antheridial hyphae induced on ♂ mycelium; reaction did not progress to subsequent stages. (*3–5*) *T. clavata* × *A. ambisexualis* ♀; successive stages of normal, fertile reaction: (*3*) attraction of antheridial hyphae of *T. clavata* to oogonial initial of *A. ambisexualis* ♀, (*4*) portion of region of intermingling of two mycelia, and (*5*) normally matured hybrid oospore. (*6*) *T. clavata* × *A. bisexualis* ♂; abortion of oogonia of *T. clavata* in region proximal to mycelium of *A. bisexualis* ♂.

esses of the ☿ were disrupted only in the region of intense antheridial production induced by the ♀. Elsewhere on the ☿ mycelium more than a normal number of antheridial hyphae were produced, but oogonial production and subsequent stages of the sexual reaction were unaffected.

Although the actual transfer of nuclei from the antheridia of *T. clavata* into the oospheres of *A. ambisexualis* and the fusion of ♂ and ♀ nuclei in the maturing oospores have not been proved, it appears most likely that such is the actual course of events.

A previous demonstration of complete sexual compatibility between species of different genera of the water molds was furnished by SALVIN (**16**) for *Achlya flagellata* × *Thraustotheca clavata*, the latter of these two homothallic species furnishing the ♂ element.

——— × *A. bisexualis* ♂: Marked induction of antheridial hyphae on the ♂ was observed at 48 hours. No attraction of the antheridial hyphae to the oogonia of the ☿ was observed. There was, however, considerable abortion of the oogonia on the ☿ mycelium in the region of intermingling (fig. 1, *6*). This effect has been most strikingly shown in the work with filtrates described below.

——— × *A. bisexualis* ♀: A very intense production of antheridial hyphae was induced on the ☿, both locally in the region of intermingling and around the periphery of the entire mycelium. This was followed by the production of a few scattered oogonial initials on the ♀ on hyphae intermingled with the antheridial hyphae of the ☿. No further reaction ensued, with the result that development of sexual organs on the ♀ never proceeded beyond the primordial stage. Many oogonial initials, scattered over the entire ☿ mycelium, failed to develop, degenerating primarily by the emptying of their protoplasmic contents into the parent vegetative hyphae.

T. primoachlya gave no discernible response in water matings with any of the heterothallic tester strains, nor was any response elicited in the heterothallic mates. The failure of response in these combinations, as will be shown below, is due to subthreshold concentrations of hormones and represents no absolute qualitative incompatibility.

The qualitative aspects of the water matings between homothallic and heterothallic species are summarized in table 2. The stage in the progressive course of the sexual reaction which was achieved in each mating is indicated by symbols referring to the hormonal mechanism in heterothallic species as diagrammed in figure 11.

The reactions described above indicate strongly the operation of hormonal co-ordinating mechanisms in homothallic plants similar to and interactive with that operative in heterothallic species. Two immediate causes of failure to ac-

TABLE 2

INTERACTIONS BETWEEN HOMOTHALLIC AND HETEROTHALLIC SPECIES IN WATER MATINGS

HETEROTHALLIC SPECIES		HOMOTHALLIC SPECIES		
		A. americana	*T. clavata*	*T. primoachlya*
A. ambisexualis	♂	NR*	A↔B	NR
	♀	Comp.†	Comp.	NR
A. bisexualis	♂	NR	A↔B	NR
	♀	A↔B‡	B↔C§	NR

* NR = No reaction.

† Comp. = Complete compatibility with normal formation and development of oogonia and maturation of oospores.

‡ A↔B = Antheridial production only; no oogonia produced.

§ B↔C = Antheridial and oogonial production but failure of further development.

See text for relative reaction intensities.

complish complete hybridization in most of the combinations are suggested by previous investigations: (*a*) specificity of hormones and (*b*) quantitative insufficiency of certain hormones. Either alone or the two in combination would result in the blocking of the sexual reaction at specific stages prior to fertilization.

The results of the water matings, while strongly indicating the existence of hormonal mechanisms in at least two homothallic species, by comparison with the known correlative mechanism in heterothallic species, do not constitute proof of the activity of hormones in the homothallic plants. Such proof would result from the demonstration of characteristic distance, or telomorphotic (**2**), responses of homothallic and heterothallic plants each to the secretions of the other under conditions prohibiting physical contact between the two. This has been accomplished by two means: (*a*) matings between homothallic plants and heterothallic tester strains in a semisolid medium in which a permeable membrane forms a physical barrier between the two interacting mycelia and (*b*) induction of characteristic sexual activities on both homothallic and heterothallic plants by filtrates derived in each case from mycelia characterized by the opposed type of sexuality.

Membrane matings

Matings in which the two mycelia were separated by interposed membranes have been made for all combinations of *A. americana* and *T. clavata* and the four heterothallic tester strains. Each contrast was made in several replicates, equally divided between the two possible patterns of inoculation in respect to the membrane.

The normal production of oogonia and antheridia on the ♀ in an agar medium possibly obscures some weak responses elicited in the older portions of these plants. The pattern of sexual activity, however, is such that intense reactions would be easily discernible. Antheridial and oogonial production is initiated in the region of inoculation only after the mycelium is well established and thereafter spreads radially from this region at the same rate as the increase in diameter of the mycelium. Thus, there is a strictly vegetative fringe, 1–2 cm. broad, at the periphery of the mycelium. Any sexual activity occurring in this normally vegetative portion is considered a positive response in the matings described below.

Assuming similar and interactive hormonal mechanisms in the two types of plants, the results of the matings of homothallic × heterothallic strains separated by permeable membranes confirm exactly the expectations based on the reactions in the water matings. The membrane matings also disclose the specific reactions more clearly and indisputably demonstrate their initiation and control by agents originating in the opposed plant and reaching the site of induced activity by diffusion across the membrane. The results of these matings are presented in summary in table 3. Pertinent information concerning specific reactions is appended to the table. Interactions between plants separated by membranes are shown in figure 2.

Although the interaction between homothallic and heterothallic species by means of diffusible hormones is indicated by the results of the water matings and is proved to be the case by the responses induced in matings with interposed membranes, these results are completely qualitative and give but little information of the details of the hormonal mechanism in homothallic plants. Comparison of responses of homothallic plants to hor-

mone-containing filtrates from heterothallic plants and vice versa with those induced in heterothallic plants by filtrates from the same species would be expected to yield substantial information about the corresponding stages in the homothallic mechanism. Unfortunately, this is only possible at the present time for the initial reaction of the sexual process, i.e., the control of the production of antheridial hyphae by means of the hormones of the A-complex.

Responses to filtrates

The responses of homothallic and heterothallic plants to filtrates containing hormones derived from plants of both sexual types show the essential similarity of the two hormonal mechanisms. At the same time, however, they also show minor and specific differences with striking clarity.

The pattern of production of antheridial hyphae under excitation by the hormones of the A-complex differs, as men-

TABLE 3

INTERACTIONS BETWEEN HETEROTHALLIC AND HOMOTHALLIC SPECIES IN MEMBRANE MATINGS

Heterothallic species	Homothallic species			
	A. americana		*T. clavata*	
	Reactions*	Fig. ref.	Reactions*	Fig. ref.
A. ambisexualis ♂	NR		A-I†	2, 4
♀	A-II B-I	2, 1 2, 2	A-IV‡ B-IV BP-IV C_1 C_2	2, 5 through 2, 11
A. bisexualis ♂	NR		A-I	
♀	A-IV§	2, 3	A-IV B-I	2, 12

* Reactions: NR, no reaction; A, production of antheridial hyphae; B, production of oogonial initials; BP, proliferative renewal of oogonial initials; C_1, attraction of antheridial hyphae to membrane; C_2, delimitation of antheridia upon membrane; I-IV, relative intensity of specific reaction.

† *T. clavata* × *A. ambisexualis* ♂: Antheridial hyphae are sparse and are confined to the older portions of the vegetative hyphae (fig. 2, 4).

‡ *T. clavata* × *A. ambisexualis* ♀: Reaction of the ♀ plant was initiated when the two mycelia were still several millimeters apart (fig. 2, 5). This reaction was observed in a mating *lacking* a membrane at a distance of 14 mm. (fig. 2, 6). Reaction of the ♀ was initiated about the time the two mycelia reached opposite lines on the two sides of the membrane (fig. 2, 7).

A process of oogonial initial proliferation, in which new initials arise from the protoplasmic reserves of the original one, prolongs during 2–3 days the receptive capacity of the oogonial initial. Proliferation is the almost inevitable result of the failure of antheridial hyphae to reach the oogonial initial during its receptive period (fig. 2, 8–10). This phenomenon has been interpreted previously in heterothallic forms as due to the lack of a specific secretion, hormone D, of contiguous, differentiated antheridia (**8**). The process of proliferation continues until a chain of spent initials is formed and the reserves of the original initial are exhausted.

§ *A. americana* × *A. bisexualis* ♀: The lack of a reaction of the ♀ in response to the very intense production of antheridial hyphae in the ♂ is apparently due to hormonal specificity blocking the reaction at this stage, since the reaction goes no further when the plants are allowed, by mating them on a plate having no membrane, to come into intimate contact (fig. 2, 3).

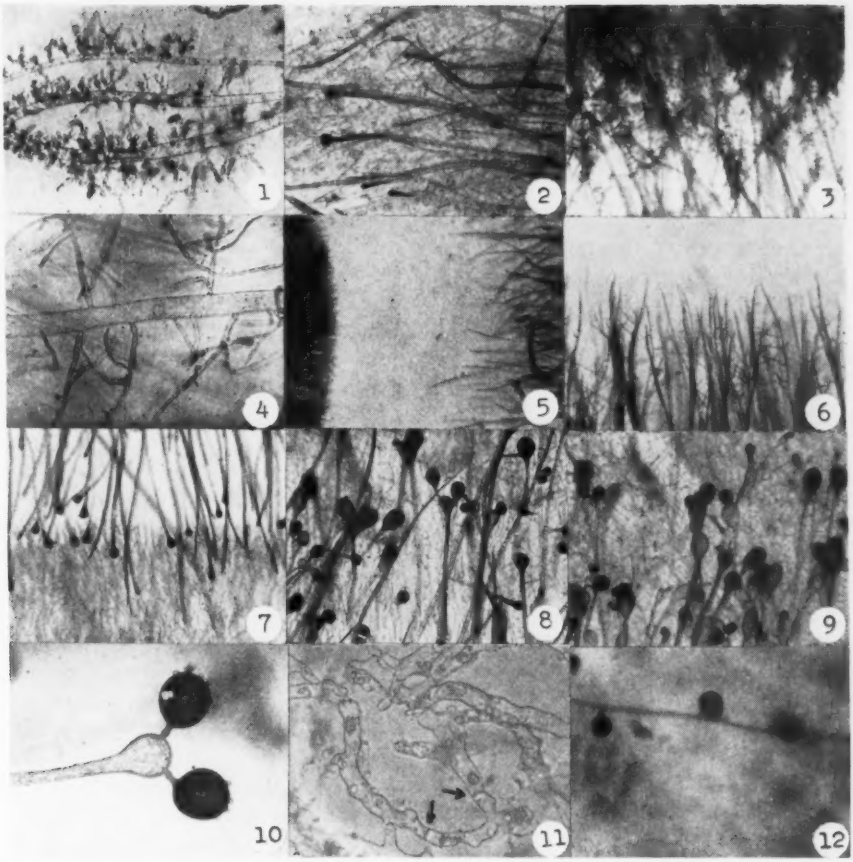

FIG. 2.—Reactions of homothallic and heterothallic species mated in agar but physically separated by interposed, permeable membranes. (*1–2*) *A. americana* × *A. ambisexualis* ♀ : (*1*) antheridial hyphae produced subterminally on vegetative hyphae of *A. americana;* (*2*) sparse production of oogonial initials on hyphal tips of *A. ambisexualis* ♀ on opposite side of membrane from antheridial hyphae shown in (*1*). (*3*) *A. americana* × *A. bisexualis* ♀ ; region of intermingling in mating in agar *without* a separating membrane, the dark region above containing thousands of antheridial hyphae of *A. americana;* no response can be seen in the ♀. (*4*) *T. clavata* × *A. ambisexualis* ♂; scattered antheridial hyphae produced subterminally on hyphae of *A. ambisexualis* ♂. (*5–11*) *T. clavata* × *A. ambisexualis* ♀ : (*5*) Intense production of antheridial hyphae on hyphal tips of *T. clavata* (*left*) before overlapping of two mycelia. (*6*) Earliest stage in telomorphotic induction of antheridial hyphal production, at a time when two mycelia were separated by distance of 14 mm. (*7*) Initiation of oogonial initial production on *A. ambisexualis* ♀. In this and two succeeding stages note antheridial hyphae of *T. clavata* beneath oogonial initials and membrane. (*8*) Continued oogonial initial production in region shown in (*7*) 24 hours later, with proliferation of an occasional oogonial initial. (*9*) Further development of oogonial initials 48 hours later, showing proliferation of most of oogonial initials. (*10*) Detail of renewal of receptive stage of oogonial initial by proliferation; some of papillae on ends of secondary initials subsequently developed into fully formed oogonial initials. (*11*) Detail of delimitation of antheridia of *T. clavata* by transverse septa (*arrows*) from antheridial hyphae in contact with membrane. (*12*) *T. clavata* × *A. bisexualis* ♀ ; oogonial initials on *A. bisexualis* ♀ ; no proliferative renewal occurred.

tioned above, in the various species studied. Two types are readily distinguishable: (*a*) Antheridial hyphae are produced, regularly spaced, on terminal regions of all hyphal tips in *A. ambisexualis* and *A. americana*. In general, an increase of hormone concentration results in the production of more antheridial hyphae per unit length of vegetative hypha as well as over a longer length of the hypha.

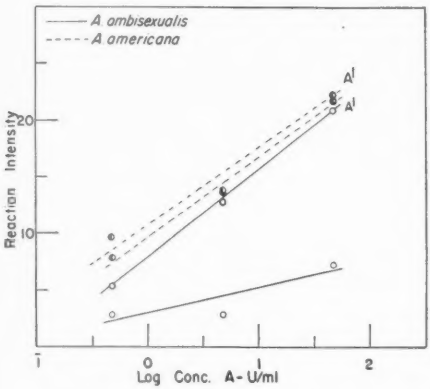

FIG. 3.—Comparison of responses of *A. americana* (*broken lines*) and *A. ambisexualis* ♂ (*solid lines*) to hormone A of *A. bisexualis* ♀ in presence and absence of hormone A^1 of *A. ambisexualis* ♂. Reaction Intensity, in this and in subsequent graphs, equals average number of antheridial hyphae produced per 3-mm-hyphal tip.

(*b*) Antheridial hyphae are produced only in localized regions, usually but not always at the tips, of certain hyphae, in *T. primoachlya*, *A. recurva*, and *A. spp.* Both the number of antheridial hyphae per affected locus and the percentage of hyphae showing a response vary directly with the concentration of the appropriate hormone. Quantitative work is feasible only with the members of the first type and is therefore limited to *A. americana* and *A. ambisexualis* ♂.

The filtrates employed were hormone A from *A. bisexualis* ♀, hormone A^2 from *A. bisexualis* ♀, and hormone A^1 from *A. ambisexualis* ♂ from the heterothallic species, and both acetone-soluble and water-soluble (acetone-insoluble) fractions of the filtrates of *A. americana*, *T. clavata*, and *T. primoachlya*. Only in the case of hormone A has a standardized unit of activity been defined; other hormone stocks have been prepared at one hundred and one thousand times the concentration of the filtrates from which they were derived and most commonly have been restored to their initial concentrations for the test. Where the concentration of a hormone is not specifically designated, it is equal to that of the original filtrate from which it was taken.

REACTIONS OF HOMOTHALLIC PLANTS TO HORMONES OF THE A-COMPLEX FROM HETEROTHALLIC STRAINS.—The quantitative response of *A. americana* to hormone A alone was in most cases almost indistinguishable from that of *A. ambisexualis* ♂ in the same concentrations of hormone A *plus* an optimal concentration of A^1. Unlike in the response of the heterothallic ♂ strain, the addition of A^1 to A had an insignificant effect on the response of the ☿. A comparison of the reactions simultaneously induced in the two plants by hormone A with and without added A^1 is shown in the accompanying graph (fig. 3), in which reaction intensity, expressed as the average number of antheridial hyphae per 3-mm. hyphal tip (ordinates), is plotted as a function of log concentration of hormone A (abscissae). There is considerable day-to-day variation in the quantitative reaction of both plants to hormone A, but, on each of numerous occasions when the two have been tested simultaneously, the agreement between the two has been extremely close.

An interesting phenomenon, repeatedly observed in practically all homothallic species, occurs in the presence of

relatively high concentration of hormone A. Oogonial initials at all stages of their development prior to the delimitation of oogonia by the formation of basal septa appear to be but partially differentiated as ♀ sexual organs and almost invariably lose their ♀ potentialities when hormone A is added. This may take place in either of two ways: by the drainage of the protoplasmic contents of the initial back into the vegetative hypha or by the outgrowth of antheridial hyphae from the oogonial initial itself (fig. 4, *1*). Upon the delimitation of the oogonium its differentiation as a ♀ sexual organ would seem to be final, and its further development is normal. Figure 4, *2* shows two ♀ organs 6 hours after the addition of 5000 AU/ml; the one on the right, undelimited, has produced a single, as yet short, antheridial hypha; the other, somewhat older and apparently delimited about the time of the addition of the hormone, is developing normally with the reorganization of its protoplasmic contents in oosphere formation. Figure 4, *3* shows an equally striking case in *T. clavata* the day following addition of 50 AU/ml (a concentration slightly below the threshold for complete inhibition of oogonial development in this species); the normal development of the three oogonial initials on the right (*arrows*) has been interrupted, and branches of an antheridial hypha originating from one of them are being attracted to two younger, normally developing oogonial initials near by.

This reaction is the most widely observed effect of the A-complex hormones on oogonial development. One other effect, brought about by hormone A^1 and limited to three homothallic species, will be described later.

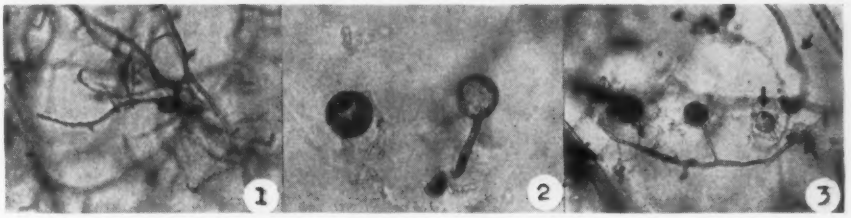

FIG. 4.—Redifferentiation and degeneration of oogonial initials of homothallic species induced by high concentrations of hormone A. (*1–2*) *A. americana:* (*1*) Growth of antheridial hyphae from oogonial initial 12 hours after addition of 50 AU/ml. (*2*) Normal development of delimited oogonium (*left*) and redifferentiation of undelimited oogonial initial (*right*) 6 hours after addition of 500 AU/ml. (*3*) *T. clavata;* redifferentiation of three oogonial initials (*arrows*) with branches of antheridial hypha originating from one initial being attracted to two normal oogonial initials (*left*) formed subsequent to addition of 50 AU/ml; photomicrograph taken 24 hours after addition of hormone.

A. americana gives a positive reaction to hormone A^2, and, as was the case with hormone A, the response follows a pattern similar to that of *A. ambisexualis* ♂ when A^1 is not limiting. Simultaneous reactions of the two plants induced by A^2 in the presence and absence of added A^1 are shown by the curves in figure 5, in which the reaction intensity is plotted against concentration of A^2 in logarithmic increments. The pattern of the response to A^2 on the day this test was performed (January 30, 1950) was quite atypical for both plants, the reaction intensity typically being a type of exponential rather than a logarithmic function of the concentration of A^2. Although the ex-

act pattern for this day was atypical, these were the only simultaneous tests made of the two plants under these conditions, and the results adequately demonstrate the similarity of the responses. The effect of added A^1 on the response of the ♀ to A^2 differs strikingly from its effect on the ♂, inhibiting rather than augmenting the response, and also differs from its relative lack of effect on the ♀ in combination with hormone A (fig. 3).

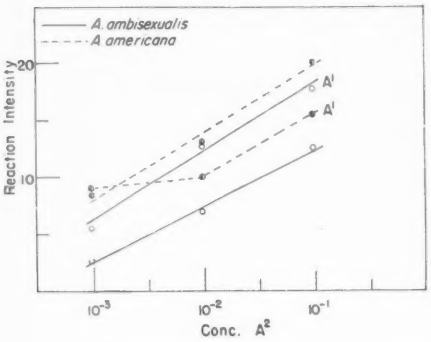

FIG. 5.—Comparison of responses of *A. americana* (*broken lines*) and *A. ambisexualis* ♂ to hormone A^2 of *A. bisexualis* ♀ in presence and absence of hormone A^1 of *A. ambisexualis* ♂.

Tests to determine the effects on *A. americana* of A^2 and of $A^2 + A^1$ in combination with hormone A gave very ambiguous results. Although there were slight differences in the series containing various mixtures of the two water-soluble hormones (A^1 and A^2) in addition to hormone A, such differences were inconsistent and of doubtful significance. On the basis of these preliminary tests it would appear that in such mixtures of hormones of the A-complex the number of antheridial hyphae produced on the ♀ is chiefly determined by the concentration of hormone A. Minor but significant effects on the reaction by A^1 and A^2 could probably be demonstrated in larger samples where errors could be markedly decreased.

The production of antheridial hyphae in other homothallic species under the stimulation of hormones of the A-complex from heterothallic forms follows, in general, that in *A. americana*, although there are conspicuous quantitative species-specific differences in addition to the characteristic localization of antheridial hyphae on all other homothallic species studied.

T. clavata is typical of those species showing localized antheridial production. The effects on this species of hormone A at various concentration levels and of hormones A^1 and A^2, each alone at a single concentration, are shown in the series of photomicrographs reproduced in figures 6 and 7. The number of antheridial hyphae varies directly with concentration of hormone A; the number of affected loci also increases with concentration except in the highest level tested, 5000 AU/ml, where the affected loci are restricted almost exclusively to relatively few hyphal tips (fig. 6, *1–7*). Each such tip, however, produces a very large number of antheridial hyphae, often exceeding a hundred per single tip (fig. 6, *8*). High concentrations of hormone A affect oogonial development as in *A. americana* (fig. 4, *3*). Hormone A^2 alone induced the production of antheridial hyphae in greater numbers than shown by the control (fig. 6, *9*) but appeared to have little, if any, effect when added to effective concentrations of hormone A. Hormone A^1 had no discernible effect on antheridial hyphal production, neither inducing their formation alone nor affecting the reaction in combination with A or A^2.

Hormone A^1, however, exhibits a very striking effect on oogonial production in *T. clavata* and two other homothallic species while having no effect on the other homothallic forms tested. Following addition of A^1 to these plants the produc-

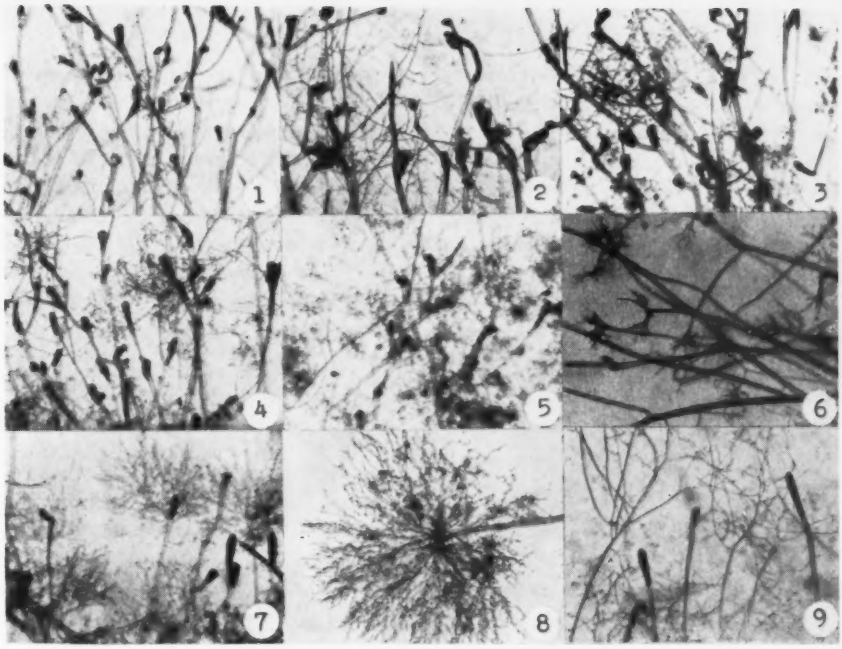

FIG. 6.—Response of *T. clavata* to hormones of A-complex (see also fig. 7, *1*, *2*). (*1*) Control plant in water. (*2–8*) Antheridial hyphal production in increasing concentration of hormone A of *A. bisexualis* ♀: (*2*) 0.5 AU/ml at 24 hours, (*3*) 5 AU/ml at 24 hours, (*4*) 50 AU/ml at 24 hours, (*5*) 500 AU/ml at 24 hours, (*6*) 5000 AU/ml at 6 hours, (*7*) 5000 AU/ml at 24 hours, and (*8*) detail of hyphal tip bearing more than one hundred antheridial hyphae, 5000 AU/ml at 24 hours. (*9*) Response to hormone A^2 of *A. bisexualis* ♀ at concentration ten times that of original filtrate.

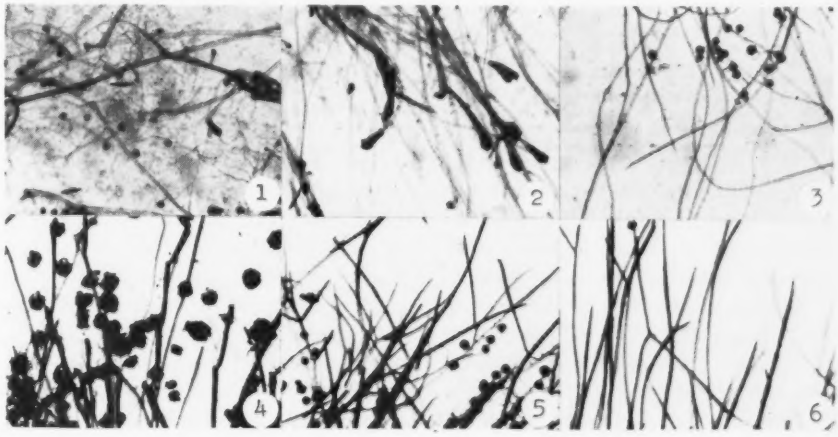

FIG. 7.—Suppression of oogonial production in three homothallic species by hormone A^1 of *A. ambisexualis* ♂. (*1–2*) *T. clavata:* (*1*) normal oogonial production on 7-day-old plant in water and (*2*) sexually sterile mycelium of same age in hormone A^1 5 days after addition of hormone. (*3–4*) *A. sp.* isolate *1.1;* normal plant in water (*3*) and sexually sterile plant in hormone A^1 (*4*); in this species zoosporangial production was markedly increased by addition of hormone. (*5–6*) *A. sp.* isolate *2;* normal plant in water (*5*) and sexually sterile plant in hormone A^1 (*6*).

tion of oogonial initials is halted, and it does not resume so long as the plants are exposed to the hormone. Photomicrographs contrasting normal and hormone A^1-treated plants of these three homothallic species are reproduced in figure 7.

The remaining available homothallic species have been tested together with *A. americana* and *T. clavata* in a series of solutions as shown by examination on the fifth day following treatment. Without exception the homothallic plants showed increased production of antheridial hyphae in response to hormone A alone. Hormone A also caused the immediate degeneration or redifferentiation of oogonial initials in most species, particularly at a concentration level of 5000 AU/ml.

TABLE 4

REACTIONS OF HOMOTHALLIC SPECIES TO HORMONES OF A-COMPLEX

Homothallic species	Test Solution														
	5000 A			50 A			50 A +A^1 and A^2			A^1			A^2		
	Effect*			Effect*			Effect*			Effect*			Effect*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
T. clavata	+++	Ø	N	++	Ø	N	++	Ø	N	−	N	O	+	N	N
T. primoachlya	+	Ø	...	++	N	...	++	N	...	−	N	...	−	N	...
A. americana	+	Ø	O	+++	O	O	+++	O	O	−	N	N	+	N	N
A. recurva	+	Ø	N	−+	O	N	−+	O	N	−	N	N	−	N	N
A. spp. 1.1	+++	O	O	++	N	N	+	N	N	−	N	O	+	N	N
2	−+	N	O	−+	N	N	+	N	N	−	N	O	−	N	N
51	+	N	N	−	N	N	−	O	N	+	N	N	+	O	N
86	++	O	A	−+	N	N	−	N	N	+	N	N†	−+	N	N
109	++	O	O	−+	N	N	+	N	N	−	N	N	−	N	N
120	++	Ø	O	−+	N	N	+	N	N	+	N	N	+	N	N
187	+	N	O	−+	N	O	−+	N	N	−	N	N	−+	N	N
A. sp. Page	+	Ø	A	−	Ø	N	−	Ø	A	−	N	N	−	N	N

* Effects: *A*, relative production of antheridial hyphae within 6 hours; *B*, effect upon oogonial initials within 6 hours; Ø = degeneration or redifferentiation (see text); N = no effect; *C*, effect on oogonial production and development during 5 days; O = no oogonia formed after treatment; A = oogonia formed but aborted; and N = no effect, oogonia normal.

† Greatly increased oogonial production.

five solutions containing hormones A, A^1, and A^2 as shown in table 4. Three effects were observed and recorded for each species in each of the five test solutions: *A*, the production of antheridial hyphae within 6 hours after the addition of the test solutions, the intensity of the reaction being roughly graded by +, ++, etc.; *B*, the effect within 6 hours on oogonial initials formed prior to the addition of the test solutions; and *C*, the effect on oogonial production and behavior subsequent to the addition of the test

In six of the eleven species examined on the fifth day, no oogonia were formed after the addition of 5000 AU/ml, while in the five remaining species oogonia developed; of these latter, the oogonia of two species aborted prior to oospore maturation. Hormone A at 50 U/ml brought about degenerative changes in oogonial initials in four species. Hormones A^1 and A^2 alone caused some little induction of antheridial hyphae and in a few cases brought about degenerative changes in oogonial initials or in oogonia. The re-

sponses elicited by the solution containing all three hormones were for the most part explicable by the separate activities of the three hormonal components. The outstanding exception to this generality is shown by *T. clavata* and *A. sp.* isolates *1.1* and *2*, each of which produced no oogonia following treatment with A^1 alone but produced normal functional oogonia in the mixture. It would appear here as if a specific effect of A^1 on oogonial production were inhibited by either A_1 or A^2.

REACTIONS OF HETEROTHALLIC ♂ STRAINS TO FILTRATE FRACTIONS FROM HOMOTHALLIC SPECIES.—In a series reciprocal to that described above, ♂ strains of *A. ambisexualis* and *A. bisexualis* were tested in acetone-soluble and water-soluble fractions of filtrates from *A. americana*, *T. clavata*, and *T. primoachlya* concurrently with hormones of the A-complex of heterothallic strains as controls. A preliminary series indicated that both acetone-soluble and water-soluble fractions, alone and in combination, from each of the three homothallic species gave positive results on each of the two ♂ strains. Furthermore, the intensity levels of the reactions induced by the filtrates of the three homothallic plants were approximately the same. The filtrate fractions from only a single species, *T. clavata*, were therefore used in the quantitative series to determine the reaction of a heterothallic ♂ strain to the secretions of a homothallic plant.

The reaction of *A. ambisexualis* ♂ to the acetone-soluble fraction of *T. clavata*, plotted in figure 8 as a function of log concentration, strongly resembles the response of this plant to hormone A. The results of these tests are quite unambiguous, although the response was weak even at the highest concentration of the fraction which was not toxic to the test plants.

The effects of the addition of hormones A^1 and A^2 to the acetone-soluble fraction are shown by the two upper solid curves in figure 8. The slight increase resulting from the addition of A^1 is of doubtful significance. The effect of A^2 added in a concentration of 10^{-2} (see fig. 5 for the appropriate control reaction for A^2 alone), on the other hand, is very pronounced,

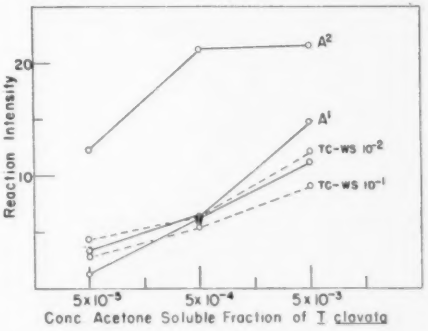

FIG. 8.—Response of *A. ambisexualis* ♂ to graded concentrations of acetone-soluble fraction of filtrate of *T. clavata* alone (*unlabeled solid line*) and in combination with hormone A^1 of *A. ambisexualis* ♂, hormone A^2 of *A. bisexualis* ♀, and two concentrations of water-soluble fraction of filtrate of *T. clavata* (*broken lines, TC-WS*).

the reaction of the combined hormone and filtrate being in each case higher than the sum of their separate activities.

The water-soluble fraction alone at concentrations of 10^{-1} and 10^{-2} (equivalent to 10× and 1× the original filtrate, respectively) induced reaction intensities of 5.7 and 3.6. Even these low reaction intensities, induced in plants which have never been observed to produce antheridial hyphae in hormone-free solutions, demonstrate clearly the secretion by *T. clavata* of a second antheridial-hyphal-inducing activity. The difference between the reactions at the two concentrations is probably significant.

The water-soluble fraction of *T. clavata* shows but little additive effect when combined either with hormone A or with the acetone-soluble fraction. These combinations are shown by the dotted curves in figures 8 and 9. The control curves, particularly in figure 9, indicate that the water-soluble fraction is not comparable with either hormone A^1 or A^2 alone. The effects of mixtures of A^1 and A^2 in the presence of A have not been sufficiently explored to compare these with the reaction given by the water-soluble fraction of *T. clavata*.

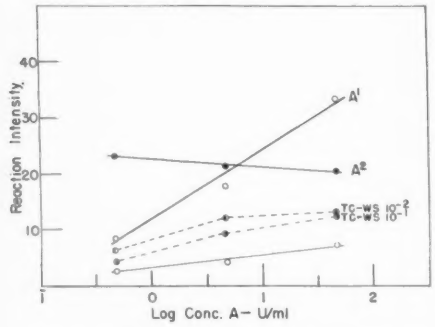

FIG. 9.—Effect of water-soluble fraction of filtrate of *T. clavata* in two concentrations (*broken lines TC-WS*) as compared with effects of hormones A^1 and A^2 on response of *A. ambisexualis* ♂ to hormone A of *A. bisexualis* ♀.

Quantitative evaluation of the reactions induced in *A. bisexualis* ♂ by the filtrate fractions of the various homothallic species is not feasible with existing methods. In general, the results were comparable with those for *A. ambisexualis* ♂, each of the two filtrate fractions from each of the three homothallic species, with a single exception, inducing the production of antheridial hyphae. Typical reactions in *A. bisexualis* ♂ are shown in figure 10, the test solution for each reaction being identified by the species listed at the top of the grid and the filtrate fraction indicated at the left. The control series at the bottom shows typical responses to hormone A alone and serves as a basis of comparison for the other reactions. The varying reactions induced by the several filtrate fractions indicate either quantitative differences in hormonal secretions of the three source plants or species-specificities of their hormones which are reflected by varying sensitivity of the test plant. One effect shown in this series of tests is of particular interest: strong responses were induced by 50 U/ml of hormone A as well as by the water-soluble fraction from *T. clavata*, whereas no antheridial hyphae were produced when the two active solutions were combined at the same concentrations (fig. 10, *10*, *4*, and *7*, respectively). In this one case two separately active secretions would appear to be mutually inhibitory in this particular ♂ strain. No comparable effect was seen in *A. ambisexualis* ♂ in another aliquot of the identical test mixture, nor was it shown by *A. bisexualis* ♂ in the same mixture when diluted by a factor of ten.

Discussion

Two significant facts are immediately obvious from the experimental findings set forth above: (*a*) a hormonal mechanism co-ordinating the sexual processes of homothallic species is of common occurrence in at least two genera of the Saprolegniaceae and (*b*) a remarkable degree of inter-effectiveness of hormones exists between the homothallic and heterothallic species investigated. From these two experimentally established facts the following possibility may be postulated: that homothallic plants possess a hormonal mechanism similar in its broader characteristics to that previously demonstrated in heterothallic species, the homothallic combining in a single mycelium the separate but interacting

chemical activities of ♂ and ♀ strains of heterothallic species.

In order to determine the extent to which this hypothesis can be substantiated by the results of the present study, it is necessary to make a rather detailed comparison between the reactions observed in homothallic-heterothallic contrasts with those obtaining between ♂ and ♀ strains of heterothallic species where the hormonal mechanism can be subjected to more direct scrutiny. The pertinent information concerning the mechanism is scattered, unfortunately, throughout the earlier papers in this series and cannot be reviewed here in the

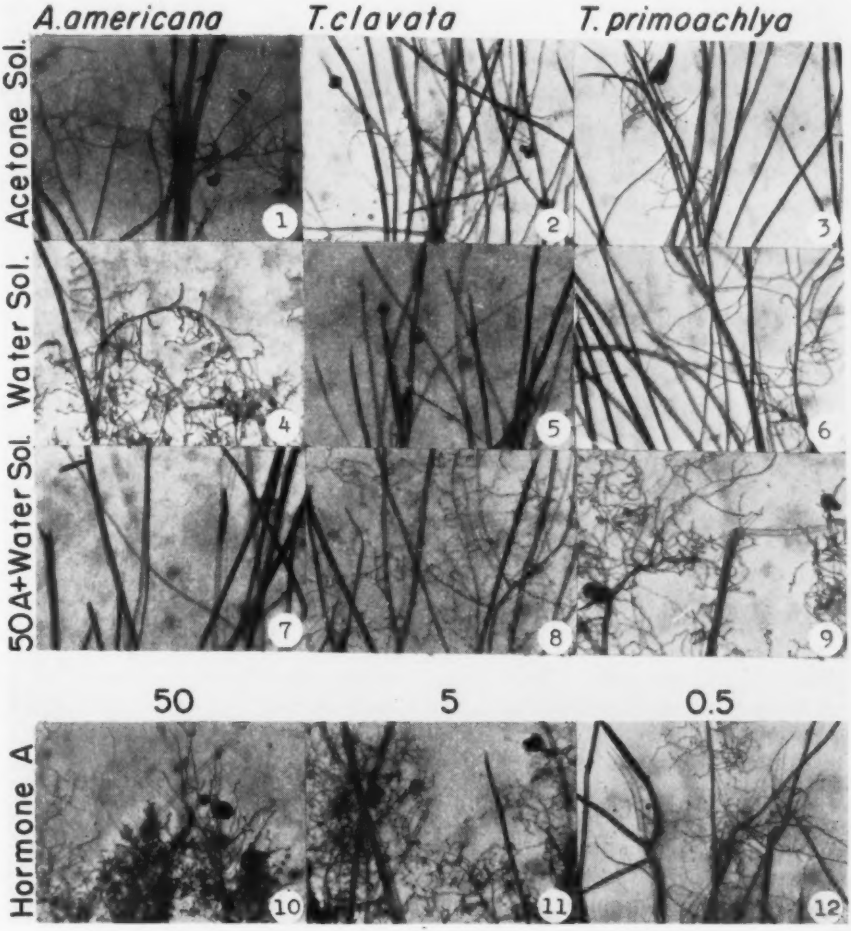

FIG. 10.—Response of *A. bisexualis* ♂ to acetone-soluble (*1–3*) and water-soluble (*4–6*) fractions of filtrates of *A. americana*, *T. clavata*, and *T. primoachlya*. (*10–12*) Control series, responses to 50 U/ml (*10*), 5 U/ml (*11*), and 0.5 U/ml (*12*) of hormone A of *A. bisexualis* ♀. (*7–9*) Responses in series combining water-soluble fractions of three species with 50 U/ml of hormone A

detail adequate for the necessary comparison. The more important facts of the heterothallic mechanism are shown in the diagrammatic scheme given in figure 11 and reproduced here as a point of reference (14).

In the water matings between homothallic species and strains of heterothallic species three types of reactions were observed: (*a*) complete compatibility, (*b*) partial or limited compatibility, and (*c*) complete incompatibility. Each of these types of response is subject to interpretation on the basis of known hormonal effects in heterothallic forms.

Complete compatibility—i.e., mating of the two plants with the production of hybrid oospores—can only result through the secretion of specifically required hormones by each of the two plants at the proper times and in sufficient quantities to induce the formation and required responses of the characteristic sexual organs of each. Thus, in the matings of *A. ambisexualis* ♀ × *A. americana* and *T. clavata* the secretion by each homothallic plant of at least two of the four ♂ hormones, B and D, is essential to the reaction. Likewise, the response of the ☿ to hormones A and C from the ♀ is essential. Of the entire complex of sexual hormones, therefore, a minimum of four must be involved for the interaction to proceed to completion. The complete compatibility shown here would further indicate the close chemical similarity, or even the identity, of the pertinent hormones of the three species involved in these matings.

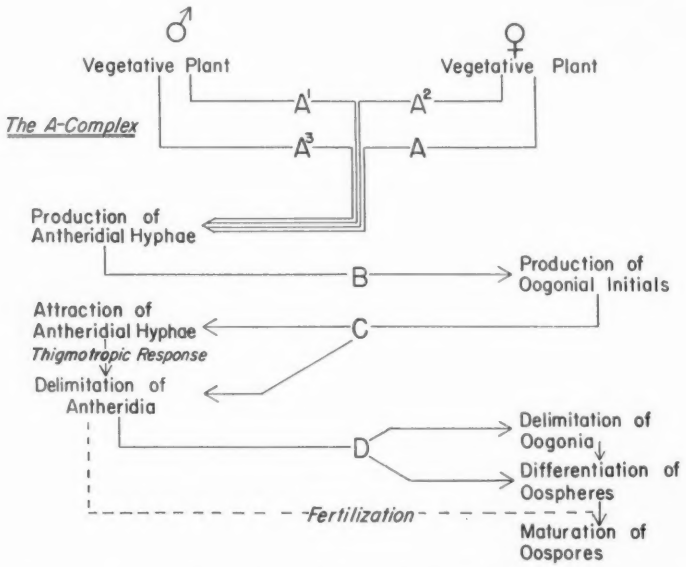

FIG. 11.—Summary diagram of hormonal correlative mechanism of sexual process in heterothallic species of *Achlya*. A detailed list of effects of A-complex hormones is given in text. (From RAPER [14].)

Partial or limited compatibility, in which the sexual process is interrupted at some stage prior to the formation of oospores, can result either from hormonal specificity or from quantitative inade-

quacy of a particular hormone. Two distinct patterns of partial or limited compatibility are represented in the matings listed in table 2. The only response induced in three matings is the production of antheridial hyphae, the mechanism of interaction being blocked at this point with the failure of further development, e.g., *A. americana* × *A. bisexualis* ♀, *T. clavata* × *A. ambisexualis* ♂, and *T. clavata* × *A. bisexualis* ♂. The reaction proceeds normally in one mating through antheridial hyphal *and* oogonial initial production after which the correlative mechanism fails, thus preventing further normal progression, e.g., *T. clavata* × *A. bisexualis* ♀. Close counterparts of these two limited response patterns have been described (**8**) in the reciprocal matings between *A. ambisexualis* and *A. bisexualis;* the former corresponding exactly to the reaction in the mating *A. ambisexualis* ♂ × *A. bisexualis* ♀, the latter corresponding, though less closely, to that in the mating *A. ambisexualis* ♀ × *A. bisexualis* ♂. It is not possible to determine from the present data which of the two probable causes given above produces the observed effect in any specific mating.

Complete incompatibility—no response in either of the two mated plants—must result either from complete hormonal specificity or from quantitative inadequacy of the hormone or hormone complex initiating the first stage of the sexual reaction. In the matings *A. americana* × *A. bisexualis* ♂, *A. americana* × *A ambisexualis* ♂, and all the matings of *T. primoachlya* except that with *A. ambisexualis* ♀, quantitative hormonal inadequacy is the immediate cause of the failure to react. This is shown by the positive response of both ♂ plants to the filtrates of both homothallic species and by the response of *T. primoachlya* to high levels of hormone A of *A. bisexualis* ♀.

No filtrate tests have been made which have any bearing on the mating *T. primoachlya* × *A. ambisexualis* ♀. It is possible, however, that hormonal specificity is the true basis for quantitative deficiency in each case, because the most probable effect of a slight change in the chemical nature of a particular hormone would be to increase the threshold at which its specifically induced response becomes possible. It thus might be argued, by extrapolation from the results observed in compatible matings, that if the precisely proper hormones were secreted, their concentrations would be adequate.

The results of the matings having membranes interposed between the two partners serve chiefly to prove that diffusible substances are the actual agents initiating and regulating the interaction of homothallic and heterothallic plants.

The responses of homothallic strains indicate both striking similarities and differences between the two hormonal mechanisms. It has been shown previously (**14**) that the intensity of the production of antheridial hyphae is dependent upon the presence and proportionality of no less than four substances: A, A^1, A^2, and A^3. The interrelationships of the various hormones are such that the number of antheridial hyphae produced is: (*a*) a logarithmic function of concentration of A, (*b*) an exponential function (of the type $y = e^{ax^k}$) of A^2, (*c*) a linear function of A^1 in the presence of A, (*d*) increased by A^1 in the presence of A^2 (the exact pattern of effect as yet undetermined), (*e*) roughly a logarithmic function of A^2 in the presence of A or in the combined presence of A and A^1, and (*f*) decreased by A^3 in the presence of A or A^2 alone or in the combined presence of A and A^1.

The responses of *A. americana*, the only homothallic species in which quantitative studies were attempted, follows this pattern in only two instances (figs. 3, 5), its reaction to A and A^2; but even in these it differs in that the reactions parallel those of *A. ambisexualis* ♂ in the presence of added A^1. In one case, with A, added A^1 has no effect; in the other, with A^2, it inhibits the reaction significantly. Further discrepancies are shown when A and A^2 are used in combination, added A^2 having no significant effect. Although indicating similar response mechanisms for the induction of antheridial hyphae in homothallic and heterothallic species, these results clearly show that the two are not identical in detail. In *A. americana*, hormone A serves the same function as in the ♂ strain, A^2 has one common activity in inducing the formation of antheridial hyphae but does not augment the activity of A, and A^1 has been demonstrated to lack its usual augmentative effect and to be effective only in the inhibition of A^2.

A similar interpretation best explains the results of the reciprocal tests in which *A. ambisexualis* ♂ was tested quantitatively in the filtrate fractions of *T. clavata* (figs. 8, 9). The acetone-soluble fraction contains activity, and by the shape of the response curve of this fraction alone its activity would appear to be attributable to hormone A. That it is a quite different hormone A from that secreted by the ♀ heterothallic, however, is shown by the lack of a strongly augmented effect when A^1 is added (fig. 8). The water-soluble fraction alone induced a very weak but definite response in the ♂ plant. This fact would suggest an A^2 activity in the fraction, since A^1 alone induces no production of antheridial hyphae. Its identity as A^2, however, appears less likely when the water-soluble fraction is added to hormone A and its activity compared with that of A plus A^2 from heterothallic strains (fig. 9) or when added to the acetone-soluble fraction of the ♀ (fig. 8). In both cases it has too little augmentative effect to compare with A^2 from the ♀.

These results could possibly be explained in either of two ways: (*a*) a somewhat simplified hormonal mechanism, in which hormones A^1 and A^2 are of greatly reduced importance, is operative in the homothallic species and (*b*) the mechanism of the homothallic forms is entirely comparable with that of the heterothallic species, but specificities are such that A^1 and A^2 only appear to be of lesser importance in the limited interspecific matings observed. The present data give no indication of which solution, if either, is the more probable.

Several of the homothallic species reacted strongly with ♀ strains of heterothallic species, and all showed at least weak responses to the ♀-secreted hormones which characteristically induce the initiating reaction in the ♂. None of the homothallic species, however, induced more than very feeble responses in ♂ strains of heterothallic species, while most of them elicited no response whatever. Thus, of twelve homothallic species selected at random (primarily as isolates collected as incidental to a wide search for heterothallic strains), three reacted as strong-♂'s and nine as weak-♂'s. None gave the reactions characteristic of ♀'s.

That the ♀ potentialities of each homothallic species were strong and expressible was attested by the copious production of oogonia on the mycelium of each in single culture. Possibly too small a collection has been employed to indicate the true pattern of sexual affinities of homothallic forms in nature. The following re-

lationships, however, have pertinence in any evaluation of the present data as regards the distribution of sexual affinities in nature.

Each homothallic species would appear to possess, in addition to the sexual characteristics requisite to its own hermaphroditism, a capacity for extra-mycelial sexual reaction. Also, the sexual affinities of a given isolate for such extra-mycelial reactions obviously vary both in degree and in kind depending on the sexual potentialities of the immediate mate—e.g., *T. clavata* in the several matings with the heterothallic tester strains. Furthermore, the capabilities for extra-mycelial reactions of the several homothallic species differ quantitatively among themselves.

In the two latter respects the homothallic isolates appear to constitute a series of sexual forms resembling the sexual intergrades of heterothallic species, particularly *A. ambisexualis*. In this heterothallic species seven distinct sexual strains, ranging from pure-♂ to pure-♀, have been recognized (**9, 13**); of these seven strains, five combine ♂ and ♀ inter-mycelial affinities and provide what appears to be a linear series between pure-♂ and pure-♀. If the homothallic species constitute a comparable series, the isolates which have been examined here would seem to correspond to those heterothallic intergrades lying within the range strong-♂ to weak-♂. Forms corresponding to the ♀ portion of the intergrade series are lacking, and only a more extensive study could show whether forms occur which exhibit strong-♀ extra-mycelial affinities.

Both of the ♂ heterothallic testers used in the present study were collected in England, where the only pure-♂ heterothallic strains have been found to date. The ♀ tester strains, however, were collected in this country from the same region which furnished most of the homothallic isolates employed. A different pattern of homothallic-heterothallic interrelationships might well result from the use of predominant-♂ heterothallic tester strains of less geographic (and presumably evolutionary) divergence. Also, the determination of the reactions in an extensive series of homothallic × homothallic interspecific matings would be expected to yield valuable information toward the elucidation of the pattern of extra-mycelial affinities of homothallic species.

A number of possible alternatives may thus be used to seek an explanation for the apparent skewing of the distribution of extra-mycelial affinities toward the behavior characteristics of ♂ plants. (*a*) The basic hormonal mechanisms of homothallic and heterothallic species may differ in such manner that interactions like those described above proceed along certain lines much more readily than along others. By comparison with heterothallic species this would appear to be unlikely. (*b*) Though the basic mechanisms in the two cases may be quite similar, hormonal specificities, particularly as regards the response sensitivities of the ♂ tester strains, could be such that only the observed responses would be possible in the particular contrasts employed. (*c*) Finally, the extra-mycelial capacities of the tested homothallic species may be precisely as they appear here, and a comparable collection from another, and widely separated, locality would reveal predominantly ♀ homothallic types. The distribution of the several strains of *A. ambisexualis* appears to follow such a pattern, ♀'s of varying potencies predominating in this country and ♂'s predominating in England.

Obviously, the present data are inade-

quate to resolve a puzzle of this magnitude. Extensive investigations along the several lines of study indicated above would be essential for a more realistic approach to the problem than is further speculation.

Another important aspect of extramycelial activity was pointed out by SALVIN (**16**) in connection with intergeneric compatibility; the present, confirming demonstrations of interspecific and intergeneric matings serve to emphasize its significance.

The occurrence of interspecific and even intergeneric matings, with the same degree of intensity as between ♂ and ♀ strains of the same species, offers at least a possible explanation for the extreme apparent lability of taxonomic characteristics and the obscurity of phylogenetic relationships throughout the group. The significance of the demonstrated compatibility between species, which may be but distantly related, can, of course, only be proved by the germination of the oosporic products of such interactions and by a careful analysis of the pattern of segregation. Even in the event that the oospores of such interspecific matings cannot be germinated and do not germinate in nature, the interaction between species could still be of decisive importance in nature. On a bit of natural substrate, taken directly from a body of fresh water, many species, each a depauperate mycelium comprising one or at most a few hyphae, occur crowded together. The sole means of initiating and co-ordinating a sexual reaction in homothallic species under natural conditions, and of providing the only stage in the life-cycle capable of withstanding adverse environmental conditions, may well be the co-operatively achieved local concentration of sexual hormones. That the oospores of homothallic species play an important role in the survival of these forms is indicated by the recent demonstration by ZIEGLER (**17**) of the germination of oospores of twenty-four homothallic species chosen at random.

The germination of the oosporic products of interspecific and intergeneric matings, such as those described above, would obviously provide an extremely promising line of research.

The findings of the present study are of significance in that they add materially to the knowledge of the biology of a large group of commonly occurring and extensively investigated plants. The demonstration of a hormonal co-ordinating mechanism in a number of homothallic forms belonging to two genera and the remarkable degree of interaction between them and two heterothallic species would indicate a common, basic physiological pattern in the sexual reproductive processes of all members of this large group.

A number of perplexing problems of long standing would appear to be explicable in terms of the hormonal co-ordinating mechanism in homothallic forms. Chief among these are (*a*) the degenerative changes in sexual organs during the sexual process as the result either of deficiencies of certain hormones or of overabundance of others; (*b*) the secondary differentiation of sexual organs to form those of the opposite sexual characteristics; and (*c*) the failure of production of ♂ and/or ♀ sexual organs. Many equally perplexing problems pertaining to sexual characteristics of homothallic forms remain as obscure as ever, but it is believed that extended study along the lines indicated above can reduce their number appreciably.

Summary

1. A simple membrane technique is described for the study of diffusible sex-

ual hormones in matings on a semisolid medium.

2. Sexual reactions have been shown to occur regularly between mated homothallic species and ♂ and ♀ strains of heterothallic species. All possible matings between two homothallic species, *Achlya americana* and *Thraustotheca clavata*, and ♂ and ♀ strains of two heterothallic species, *A. bisexualis* and *A. ambisexualis*, reveal relationships ranging from no reaction to complete compatibility, and presumably hybridization, between the two mated plants.

3. Matings between the homothallic and heterothallic species listed above, in a semisolid medium in which interposed membranes physically separate the mated plants, prove that the initiation and coordination of the sexual interactions result from diffusible hormones.

4. Assuming similar and interactive sexual hormonal mechanisms in homothallic and heterothallic species, the results of membrane matings confirm exactly the expectations based on the reactions observed in water and agar matings.

5. The effects of hormone-containing filtrates from homothallic and heterothallic species have been reciprocally determined on species showing both types of sexuality.

6. Homothallic plants react to hormone A in a quantitative pattern similar to the response of heterothallic ♂'s to hormone A *plus* hormone A^1. Antheridial hyphal production is but little affected by hormones A^1 and A^2.

7. Degeneration and/or redifferentiation of oogonial initials results from high concentrations of hormone A in nine of twelve homothallic species investigated. Oogonial development, however, is normal if delimitation of the oogonium had occurred prior to the addition of the hormone.

8. Complete inhibition of oogonial initial production results from the addition of hormone A^1 in three homothallic species.

9. Three homothallic species, *A. americana*, *T. clavata*, and *T. primoachlya*, were each shown to secrete both acetone-soluble and water-soluble (acetone-insoluble) substances capable of inducing antheridial hyphal production on ♂ heterothallic plants. Each fraction, however, induces the reaction in quantitative patterns which preclude the identities of these hormones with the hormones of the A-complex from heterothallic species.

10. The conclusion is reached that homothallic species possess a hormonal mechanism similar in broad pattern to that previously demonstrated in heterothallic species, but that striking differences in detail, possibly only as regards hormonal specificities, obtain between the mechanisms characteristic of the two types of sexuality.

11. Each homothallic species possesses, in addition to the sexual capabilities requisite to its own hermaphroditism, affinities for extra-mycelial reactions, the kind and degree of which depend upon the sexual affinities of the immediate mate. Limited evidence is presented which indicates that the homothallic species studied range, as regards extra-mycelial potentialities, from strong-♂ to weak-♂. These correspond to the predominant-♂ intergrades of heterothallic species in that they also possess weak ♀ as well as ♂ potentialities.

12. The possible significance of interspecific and intergeneric reactions involving homothallic species in nature is briefly discussed.

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FORMATION AND LIBERATION OF ZOOSPORES IN ISOACHLYA ANISOSPORA VAR. INDICA[1]

K. S. BHARGAVA

Introduction

Two great pioneers, PRINGSHEIM and DE BARY, took the initiative in working out the details of structure and life-history of some members of the family Saprolegniaceae. Since the description of spore formation in *Achlya prolifera* in 1850 by PRINGSHEIM (**27**), the processes of sporangium development and the liberation of zoospores have been studied in various genera belonging to the family. The genera so far studied include *Achlya* (**7, 9, 12, 16, 18, 19, 26, 30, 37**), *Aphanomyces* (**9, 30**), *Brevilegnia* (**6, 10**), *Geolegnia* (**10**), *Leptolegnia* (**9, 25**), *Saprolegnia* (**5, 11, 22, 23, 29, 37, 39, 40**), and *Thraustotheca* (**32, 36, 41**). Several abnormalities have been noted by various workers (**9, 17, 24, 42, 43**).

[1] Part of a thesis approved for the degree of Doctor of Philosophy at the University of Allahabad.

Recently SALVIN (33) studied the effect of temperature on the zoospore activity of *Saprolegnia* W, *S. delica*, *Achlya flagellata*, *Thraustotheca clavata*, and *Dictyuchus* 2. A little later he reported (34) the influence of environmental variations in temperature, oxygen tension, etc., on the shape and size of the sporangia of *Brevilegnia*. He also found (35) that environmental factors partly control the successive production of the sporangia of *Thraustotheca primoachlya* and *Dictyuchus achlyoides*.

A review of the available literature shows that, although the genus *Isoachlya* is of considerable interest, the cytological details of its development have not been investigated.

The purpose of the present study was to follow the development of zoospores in living material, to study the effect of temperature and hydrogen-ion concentration on their formation, and to make a cytological investigation of the development of zoospores and their liberation in *Isoachlya anisospora* var. *indica*.

Material and methods

Single-spore cultures were prepared according to the method recommended by COUCH (10). To obtain very vigorous growth with numerous sporangia, the cultures were transferred from the agar to hemp seed in water. A small piece of agar bearing the fungus was cut from the margin of the young colony and placed in a sterilized Petri dish, a half portion of a sterilized hemp seed was put on the agar piece, and a little sterilized distilled water was added. The method devised by KLEBS (20) and later used by other investigators, which involves the sudden diminution of food supply, was also followed: the organism was grown in several rich media—peptone solution, pea broth, lentil decoction, hemp decoction, etc.—for 24 hours and then transferred to water which had been sterilized, distilled, and pretreated with a small quantity of animal charcoal.

The development of the sporangium in the living condition with and without neutral red was studied under water-immersion lens and in hanging-drop cultures, but for the behavior of zoospores the latter method only was used.

The sporangia were killed and fixed when they had reached the desired stages in development. As fixing agents Bouin's fluid (3), CLAUSSEN's chromoacetic acid (4), Flemming's weak solution modified by SAKSENA (31), and RAPER's chromic acid-formalin solution (28) were tried; the last gave the best results. The time for fixation varied from 12 to 24 hours. The fixed material was washed, dehydrated, and imbedded in paraffin in the usual manner. Sections cut 4–5 μ thick were stained on the slide with crystal violet according to Gram's technique described by COUCH and modified by SHANOR (38). Some of the material was stained in iron alum-haematoxylin and mounted en masse.

Zoospores were killed by exposure to 1% osmic acid vapors for 20–45 seconds and treated by the technique formulated by CÖTNER (8).

For the preservation of mitochondria the material was fixed in Helly's liquid and formol-sublimate (31). Sections 4 μ thick were stained with iron alum-haematoxylin in the usual manner

Observations

LIVING MATERIAL

Sporangia at first are indistinguishable from the hyphae. Indeed, every hypha is a potential sporangium. The various stages in the development of the sporangium resemble closely those already ob-

served in different species of *Achlya* (**9, 16, 18, 26**) and *Saprolengia* (**11, 37, 39**), and no additional description seems necessary. At maturity the papilla at the sporangial tip gives way, and the zoospores rush out one by one in a manner typical of *Saprolegnia*. An important point to be noted, however, is that the first spores to be liberated stay at a short distance from the mouth of the sporangium until the entire group of zoospores has emerged. All then immediately swim away. The zoospores are in a state of constant agitation during this interval which is of short duration, lasting for only 2–3 seconds. The size of the sporangium varies from 14.5 to 24.5 μ in thickness and from 99 to 163.7 μ in length.

INTRAVITAL STAINING.—The fungus was grown in peptone solution (1%) for 24 hours and was then transferred to sterilized, well-oxygenated, distilled water to which had been added 0.0001–0.01% of neutral red. Sporangia were formed and zoospores discharged only where the concentration of neutral red was less than 0.003%. In a similar experiment GUILLIERMOND (**14, 15**) found that a species of *Saprolegnia* formed sporangia only when the concentration of neutral red was less than 0.005%, while SASKENA and BHARGAVA (**32**) showed that sporangiospores in *Thraustotheca clavata* were formed and discharged with a concentration as high as 0.012% of neutral red.

A single thread bearing a sporangium initial was cut, washed with distilled water, and observed constantly in hanging-drop culture. A vacuole, colored orange-red, is seen in its center (fig. 1). A little later this axial vacuole sends off small colored extensions toward the wall of the sporangium, cutting the protoplasm into polygonal masses, devoid of any color of neutral red. In optical section the central vacuole extending from near the base to the tip of the sporangium presents a zigzag appearance (fig. 2). Precipitates are also present in the vacuole (fig. 1).

At the so-called "homogeneous stage" the color of the dye diffuses throughout the sporangium, and the identity of the colored lacunae is lost (fig. 3). In the final phase, which begins with the rounding-off of spore initials, no trace of neutral red is found in places formerly occupied by the colored lacunae, but each spore presents a single (fig. 4), though sometimes a second small colored vacuole. With the bursting of the apical papilla and escape of the zoospores, the precipitate formed inside the vacuole also comes out of the sporangium.

Each zoospore, whether swimming or encysted, usually contains one orange-colored vacuole (figs. 5, 6). In the encysted stage the shape of the vacuole is usually round, while in the swimming stages it has a cup-shaped form. Within the germinating spore the vacuole is seen extending into a canal (figs. 7, 8).

SUPRAVITAL STAINING.—The sporangia at various stages of development were irrigated with neutral red dissolved in Ringer's solution. The observations already reported under intravital staining were confirmed. In the tips of the germ tubes, besides the vacuolar canal extending from the germinating spores, tiny isolated vacuoles were seen arising *de novo*, giving rise to bigger ones by fusion.

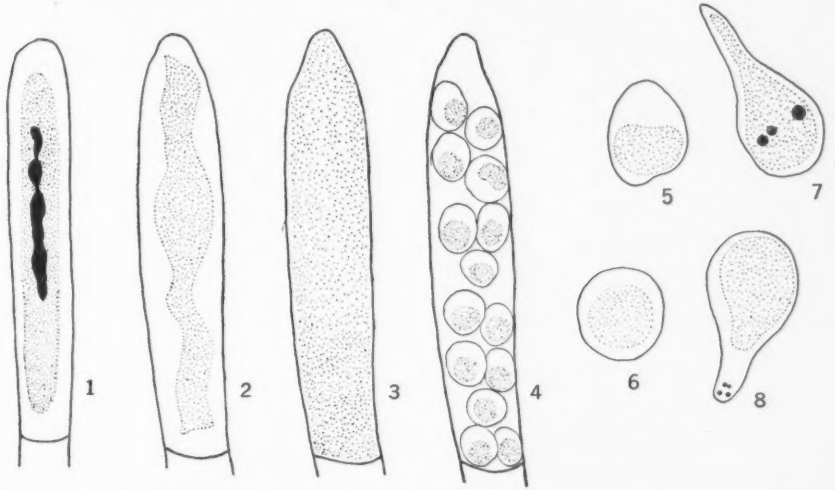
FIXED MATERIAL

NUCLEI.—Nuclear behavior during spore formation in this fungus is similar to that found in other members of the family Saprolegniaceae (**4, 9, 11, 25, 26**). In conformity with the observations of other workers no division of the nucleus has ever been observed in any stage of development of the sporangium or zoo-

spores (figs. 9–19). Although efforts were made to show them, astral rays radiating from the nuclei were not seen.

CHONDRIOME.—A tip of a hypha about to become a sporangium shows mitochondria in the form of big rods as well as of granules (fig. 20). These rods are smaller than the filamentous mitochondria of the vegetative body. They are seen in the process of fragmentation, giving rise to still smaller rods or granular mitochondria. After the delimitation of the sporangium from the main hypha, mostly granular and short, rod-shaped mitochondria are seen lying throughout the sporangium (fig. 21). These forms of mitochondria persist through all the stages in development of the sporangium (figs. 22–24). After the first division the mitochondria are aggregated around the nuclei.

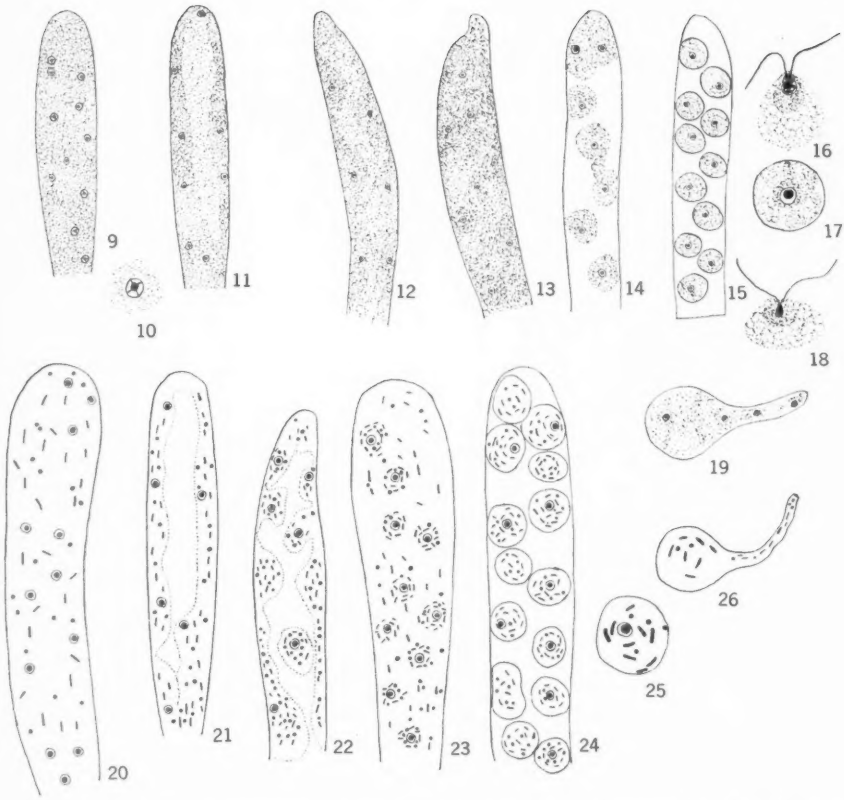
In the zoospore, mitochondria in these two forms are present and lie scattered around the nucleus (fig. 25). In the young germ tubes, rod-shaped and filamentous mitochondria are seen behind the tips (fig. 26).



FIGS. 1–8.—*Isoachyla anisospora*. Stages in development of sporangium and zoospores grown in medium supplemented with neutral red. Figs. 1–4, × 1024; figs. 5–8, × 1536. Fig. 1, young sporangium showing axial vacuole with vacuolar precipitates. Fig. 2, vacuolar extensions colored orange red, giving zigzag appearance, and spore initials. Fig. 3, sporangium at "homogeneous stage" showing diffused color of neutral red. Fig. 4, sporangium with mature zoospores, each of which has vacuole. Figs. 5–8, zoospores. Fig. 5, first swimming stage, showing cup-shaped vacuole. Fig. 6, resting condition, showing round vacuole in center. Fig. 7, germinating, showing extensions of vacuole into vacuolar canal. Fig. 8, germinating, showing vacuoles arising *de novo* at tips of germ tubes.

INFLUENCE OF EXTERNAL CONDITIONS ON DEVELOPMENT OF SPORANGIUM

TEMPERATURE.—The fungus growing in peptone solution (1%) was carefully washed, transferred to sterilized, distilled water, and kept at temperatures ranging from 5° to 35° C. At 5° C. no sporangia developed, while at 10° C. only a few were formed, and the zoospores were discharged only after 18 hours. There was an increase in number of sporangia formed at 15° C., but the time taken by the zoospores to discharge was nearly the same as at 10° C. The number of sporangia developing to maturity was



FIGS. 9–26.—*Isoachlya anisospora*. Various stages in development of sporangium and zoospores, in fixed condition; figs. 9–19 stained to show nuclei and cytoplasm, figs. 20–26, to show mitochondria. Figs. 9, 11–15, × 687; fig. 10, × 1875; figs. 16–19, 25, 26, × 1344, figs. 20–24, × 896. Fig. 9, longisection of young sporangium showing nuclei distributed irregularly throughout. Fig. 10, single nucleus of above, enlarged. Figs. 11–15, longisections of sporangia. Fig. 11, showing vacuole in center and nuclei toward the periphery. Fig. 12, at later stage showing uninucleate spore initials in optical view. Fig. 13, at "homogeneous stage" showing scattered nuclei with aggregation of cytoplasm around each. Fig. 14, showing young zoospores. Fig. 15, mature sporangium with mature uninucleate zoospores. Fig. 16–19, zoospores. Fig. 16, first swimming stage showing two cilia at narrower end. Fig. 17, resting condition showing nucleus moved toward center. Fig. 18, second swimming stage showing two cilia on side. Fig. 19, germinating. Figs. 20–24, longisections of sporangia. Fig. 20, sporangium initial showing mitochondria in form of big rods and granules. Fig. 21, young sporangium. Fig. 22, in nearly same stage as in figure 12. Fig. 23, in stage shown in figure 13, mitochondria aggregated around nuclei. Fig. 24, mature, with mitochondria in each zoospore. Fig. 25, zoospore showing nucleus and mitochondria. Fig. 26, germinating zoospore with elongated germ tube; a few granular mitochondria are seen at tip and rod-shaped ones behind them.

still greater at 20° C., and the whole process was completed within 8 hours of the transfer. A temperature of 25° C. was most favorable for the formation of sporangia and the discharge of zoospores. The number of sporangia was abundant, and the time taken by the zoospores to develop and to discharge was only $4\frac{1}{2}$ hours. Only a few sporangia were formed at 30° C., while a temperature of 35° C. inhibited their formation altogether.

HYDROGEN-ION CONCENTRATION.—Some of the carefully washed mycelium was transferred to water which had been distilled, sterilized, and adjusted to different pH values ranging from 3.5 to 10. The fungus was also grown on halves of hemp seeds in similar solutions. Sporangia developed and zoospores were discharged in all cultures with pH values of 4.5 to 9.0. Sporangia were rare in cultures with pH values 4 and 9.5, while none was formed at pH 3.5 or 10.

Discussion

A number of investigators (**1, 13, 21**) showed that in the Leptomitaceae there is a simultaneous cleavage in the sporangia. The results obtained in the present study are contrary to their observations but are in accord with those of SCHWARZ (**37**) and COUCH (**10**) who, working with Saprolegniaceae, showed that progressive cleavages start from the middle and proceed toward the periphery of the sporangium or vice versa.

Since HARTOG's excellent work (**16**) on the formation and liberation of zoospores in Saprolegniaceae, it has been well recognized that at the so-called "homogeneous stage" the plasma membrane bursts, and a part of the cell sap is expelled, leading to a shrinkage of the sporangium. It has been further suggested that the developing spores absorb water from the surrounding medium, swell, and become rounded off. The writer is of the opinion that, instead of absorbing water only, the developing spores first reabsorb the cell sap present in the sporangium. This is suggested by the appearance of color in the developing vacuole of the spore initials which do not show any color in the earlier stages. Similar results have been obtained by SAKSENA and BHARGAVA (**32**) in *Thraustotheca clavata*.

That the vacuoles in plant cells may arise *de novo* is clearly seen in *Isoachlya* also. That the spore initials, at a stage when the vacuole in the sporangium is in the form of intersecting lacunae, do not contain any vacuole is shown by the absence of neutral red. But afterward, when the spore initials begin to round off, small colored vacuoles appear and gradually increase in size. Moreover, the germinating zoospores show small vacuoles arising *de novo* at their tips. Similar results have been reported in *Saprolegnia* (**2, 14**) and in *Thraustotheca clavata* (**32**).

The granular mitochondria give rise to filamentous forms in the vegetative hyphae, and the filamentous forms fragment into small rod-shaped and granular mitochondria during the formation of the sporangium. They persist in these forms in all the later stages until the germination of the spores when they again elongate. These observations show that mitochondria are present in all parts of the fungus and are permanent elements.

Summary

1. The formation of zoospores and their liberation have been studied in *Isoachyla anisospora* var. *indica*.

2. The development of the sporangium is similar to that in *Achlya* and *Saprolegnia*.

3. When grown in a medium supple-

mented with neutral red, the young sporangium is seen with a colored axial vacuole which later on sends out extensions cutting the protoplasm into spore initials devoid of any color of neutral red. Spore initials, after passing through the "homogeneous stage," round off, forming zoospores each of which contains a colored vacuole. Each zoospore on germination gives rise to a germ tube at the tip of which tiny vacuoles are seen arising *de novo*.

4. Nuclear behavior during spore formation is similar to that found in other members of the family Saprolegniaceae.

5. Mitochondria are present as rod-shaped and granular structures in the young sporangium. They retain these forms throughout the later sporangial stages and also in the mature motile or resting zoospores. In the germ tubes rod and filamentous forms are seen.

6. The optimum temperature for the formation of sporangia lies near 25° C. They are formed in media having pH values between 4.5 and 9, although more acidic or alkaline media inhibit their formation.

The present work was carried out during the tenure of a fellowship at the University of Allahabad, and the writer is much indebted to Dr. R. K. SAKSENA for his kind guidance and encouragement throughout the course of the study. His thanks are also due to Dr. F. M. L. SHEFFIELD for kindly going through the manuscript.

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EMBRYOLOGY OF SEWARDIELLA TUBERIFERA KASH.

P. N. MEHRA AND A. L. KHANNA

Introduction

The monotypic genus *Sewardiella* was first discovered by KASHYAP (2), who gave a general morphological description of the species and placed it in the family Codoniaceae of the anacrogynous Jungermanniales. Another paper was published on mycorrhiza in this species by CHALAUD (1). MEHRA (3) reported the diploid chromosome number for the species to be eighteen. No work has been done on the development of sex organs in this interesting genus, and it is primarily with this object that the present investigation was undertaken.

Material and methods

The species has a very restricted distribution. It has so far been recorded from only two places—Mussoorie and Simla in the northwestern Himalayas at an altitude of 7,000 feet. At Mussoorie it is found in the vicinity of Woodstock College but is strictly localized, growing

only along the slopes on the Tehri road within a range of about 2 miles.

The material was collected from Mussoorie on three different occasions in the second week of July, 1945, in the third week of June, 1946, and again in the third week of August of the same year. Professor KASHYAP's collection made in September, 1932, supplied the material with fully developed sporogonia.

Fixation was done on the spot in formalin-acetic alcohol, Allen's modification P.F.B.$_{15}$ of Bouin's fixative, and Randolph's modification of Navaschin's fluid. Sections for histological study were cut 10–12 μ thick and for the development of sex organs 8–10 μ. The slides were stained with safranin in combination with gentian violet or fast green and with iron-alum haematoxylin.

Observations

HABIT.—The plants of *Sewardiella* grow in very shady places with high humidity and are generally associated with other liverworts like *Anthoceros himalayensis* Kash., *Fossombronia himalayensis* Kash., *Fimbriaria reticulata* Kash., and *E. blumeana* Needs, and with prothalli of the fern *Gymnogramme leptophylla*. They are covered by grasses, *Selaginellas*, mosses, and ferns. They never occur in an open growth and are rarely found in places which are directly exposed to sunlight.

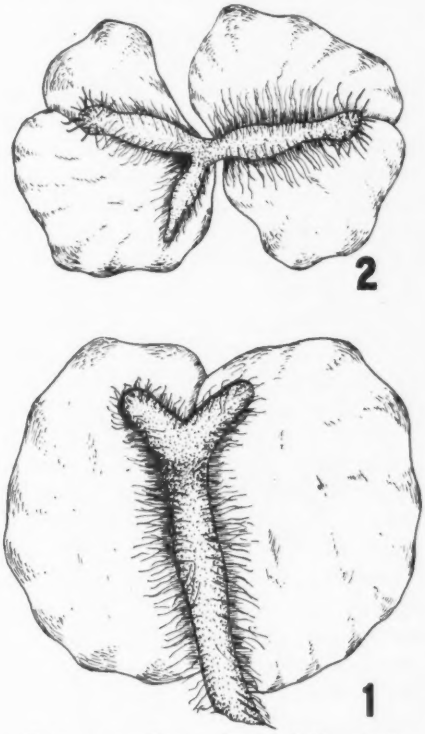
The species is strictly dioecious. Some plants remain sterile, and these are invariably more vigorous than either males or females. Among the fertile plants the females are usually larger than the males. Plants may occur singly, though frequently they grow in patches. Usually male and female plants occur in separate patches which grow side by side, though often they may be widely separated. The sterile plants appear to be as numerous as the male or female ones and are freely distributed in each patch. It is not known whether there are genetic differences. In the month of August when the female plants have been fertilized and some show sporogonia with a well-developed perianth, male plants can hardly be detected.

EXTERNAL FEATURES.—The thallus is cordate, dorsiventral, and characterized by its saddle-shaped appearance resulting from possession of a thick central midrib and two lateral, thin, flattened wings (fig. 1). Thus it is strikingly similar to the prothalli of *Gymnogramme leptophylla*. Often it may be dichotomously branched (fig. 2). The dorsal surface of the midrib bears the antheridia or archegonia.

The wings are often unequal in size and their shape variable. Although the latter is usually entire or slightly undulate, it may quite commonly be partly incised. These incisions may be shallow or deep and, in some cases, even reach the midrib. The wings are more or less ascending in the young condition, but, as the plants mature, the midrib flattens, and this puts a great pressure on the wings, which consequently become somewhat ruffled and ultimately prostrate. The midrib, which is quite thick, is flat above and projects ventrally where it bears colorless, tubular, unbranched, and smooth-walled rhizoids. There are usually two, but occasionally three to four, lax rows of dark-brown scales on the ventral surface of the midrib. These scales on the mature plant are minute, possess irregular margins, and are without appendages. They are not so well marked when the plant is young.

HISTOLOGY.—The structure of the midrib is very simple. It is twenty to twenty-five cell layers thick. The upper five or six layers are composed of large

polygonal cells with little content, while the central zone of twelve or thirteen cell layers is formed of somewhat smaller and more elongated cells with relatively dense cytoplasm. Both of these zones are photosynthetic. The lowermost zone of five to eight layers of cells is mycorrhizic. The mycorrhiza, however, is restricted to the posterior region of the midrib. There



FIGS. 1, 2.—Plants of *Sewardiella tuberifera*, ventral surface, ×4.

are no mucilage cells in the midrib, nor is there any differentiation of conducting parenchyma.

The wings are for the most part only one cell layer thick except near the midrib, where they may be two- to four-layered. There is no structural differentiation; all cells are alike and parenchymatous.

MYCORRHIZA.—CHALAUD (1) studied the mycorrhiza in the species intensively, and the present observations are in accord with the facts stated by him. The fungus is intracellular and can be seen passing from one cell to another. The hyphae are branched, coenocytic, and often somewhat swollen locally. In passing from one cell to another, the hyphae pierce through the cell wall; and passage is probably affected by the dissolution of the wall following secretion of some enzyme. Many cells are almost filled with

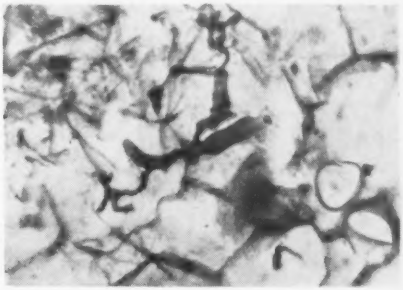


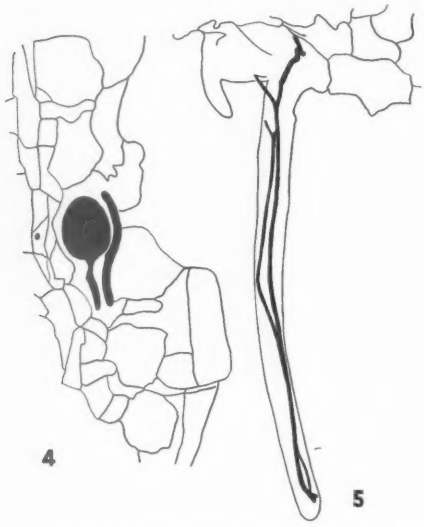
FIG. 3.—Robust hypha of mycorrhizal fungus producing a thin branch.

hyphae. In some cells the hyphae are of narrow diameter while in others they are thicker. That these hyphae are of the same fungus species is shown by the fact that the narrower hyphae arise directly from the more robust ones (fig. 3).

The infection of the tissue occurs from the soil through the rhizoids. Figure 5 shows a rhizoid with two fungal filaments within. Not infrequently three filaments may be observed. The sex organs of the fungus were not seen. There were, however, thick-walled vesicles, either attached at the ends of hyphae or encircled by them, almost completely filling up the interior of the cells in the most posterior region of the midrib (fig. 4). These vesicles are usually circular or kidney-shaped, with vacuolated cytoplasm and

numerous darkly staining granules. CHALAUD has reported the presence of numerous spores within these vesicles, but spores have not been observed by us.

APICAL CELL.—Growth of the thallus results from the activity of a prominent tetrahedral apical cell. Three cutting faces are seen in a transverse section (fig. 8), while in longitudinal section only two of these are visible (figs. 6, 7). Because there is a greater growth of the cells of the ventral region near the apex, the apical cell comes to lie in a depression on the upper surface, where it is covered by a number of upturned hairs with mucilaginous tips (fig. 7). These uniseriate hairs, several cells long, arise from the ventral surface of the thallus near the apex and probably serve in protecting the growing point against mechanical injury and drought.



FIGS. 4–5.—*Sewardiella tuberifera*, ×250. Fig. 4, hypha of mycorrhizal fungus terminating in vesicle. Fig. 5, two fungal hyphae in rhizoid.

In two cases a curious abnormality was observed. The apical cell had undergone a transverse division as seen in a longitudinal section of the thallus (fig. 9). The significance of this is not quite clear.

That the dichotomous branching of the thallus is true dichotomy is shown in figure 8, which represents a transverse section of a thallus at the growing point. Two tetrahedral cells of equal size were obviously the division product of the original single apical cell.

PERENNATION.—Perennation is accomplished by means of apical tubers as described by KASHYAP (2). They are borne on short cylindrical stalks which are continuations of the midrib. Often the stalk is forked, each branch bearing a tuber at its apex. Development results from the action of a tetrahedral apical cell as in the case of the thallus (fig. 10).

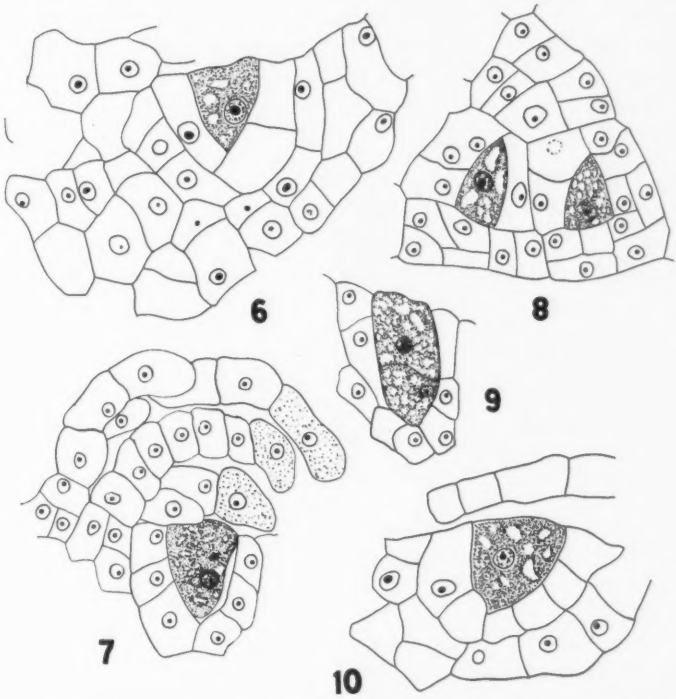
The tubers are initiated about the end of July and by October are well developed. They remain dormant during the winter and germinate during the next growing season, sometimes as early as the later half of February.

The tuber is composed of somewhat elongated parenchymatous cells. The peripheral one to three layers are mycorrhizic, although there is not so profuse development of the hyphae as in the thallus. The mycorrhizal infection may therefore be perpetuated by means of the tubers. An interesting feature is the presence of antheridia growing in acropetal succession on the tuber stalk. Presence of antheridia has also been reported by MEHRA and MEHRA (4) on the tubers of *Stephensoniella brevipedunculata*.

MALE PLANT

The antheridia are borne in acropetal succession on the upper surface of the midrib forming three or four irregular rows. Each antheridium arises in the axil of a bract which covers it; in the axils of the larger bracts, however, two or three

antheridia may develop. Consequently, bracts are scattered in the antheridial region. These bracts may be large or small, branched or unbranched, and two to four cells broad at the base. The mature antheridium is globular, attached to the

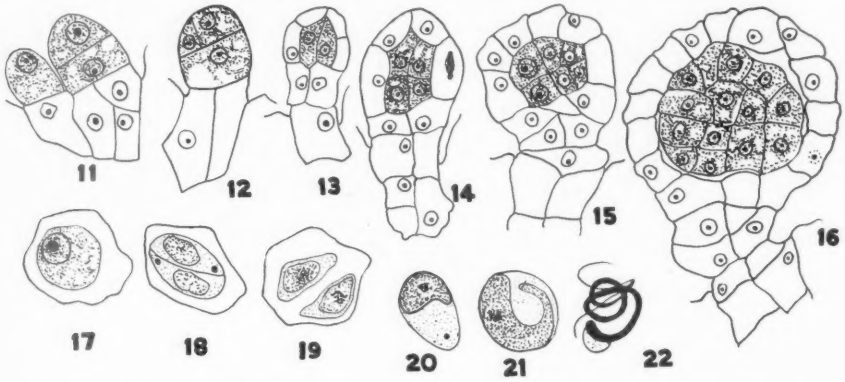


FIGS. 6–10.—*Sewardiella tuberifera* apical cells. Figs. 6, 7, longitudinal section of apical cell of thallus. Fig. 8, transverse section of apical region of thallus showing two apical cells. Fig. 9, longitudinal section of apical cell divided by transverse wall. Fig. 10, longitudinal section of apical cell of tuber.

midrib by a short multicellular stalk, and invariably inclined forward.

DEVELOPMENT OF ANTHERIDIUM.—The development agrees in all respects with that in other anacrogynous Jungermanniales. The antheridium originates from a papillate initial projecting above the thallus surface. It divides by a transverse wall whose plane is somewhat above the general surface of the thallus (fig. 11, *left*). This results in the formation of a basal cell which remains within the thallus and an outer antheridial initial which projects considerably. The outer cell has a conspicuous nucleus and deeply staining cytoplasm. This cell divides by a transverse wall into two equal halves, an outer primary antheridial cell and an inner primary stalk cell (fig. 11, *right*). Next a vertical wall appears in the antheridial cell (fig. 12). This is followed by a vertical wall in the stalk cell which may be parallel or at right angles to the vertical wall of the antheridial cell. In each of the two primary antheridial cells two periclinal walls at right angles to

each other are laid down so that the antheridium at this stage consists of four peripheral cells inclosing two primary spermatogenous cells which stain more deeply. Figure 13 shows this condition of the spermatogenous tissue, but the wall cells have been further divided by a transverse wall. Further growth of the antheridium results from the division of the primary spermatogenous cells. First a transverse wall in each cell results in four cells (fig. 14); repeated divisions then occur in all directions (figs. 15, 16). At the same time the wall cells undergo anticlinal divisions, thus increasing the single-layered wall of the antheridium to accommodate the spermatogenous tissue within.



FIGS. 11–22.—*Sewardiella tuberifera*. Figs. 11–16, stages in antheridial development. Fig. 17, sperm mother cell. Figs. 18, 19, diagonal division of sperm mother cell to form spermatids. Figs. 20, 21, stages in sperm development. Fig. 22, mature sperm.

The spermatogenous tissue is broadly divided into compartments which are lineally descended from the early formed spermatogenous cells. In each compartment all the cells are at the same stage of mitotic activity at any one time, while those in any other compartment may be at another. The same feature has been observed in *Stephensoniella brevipeduncu-lata* by MEHRA and MEHRA (4) and is probably true in all liverworts. It is curious that the same factor or force governs the mitotic activity of all cells in any one compartment and has nothing to do with those in the other.

Some stages of sperm development have been observed. The contents of the sperm mother cell round up and retract from the cell wall (fig. 17). Division of the large nucleus is followed by a diagonal cleavage of the cytoplasm, giving rise to two spermatids in the usual fashion (figs. 18, 19). The two spermatids at first lie closely appressed to each other but later separate. Each spermatid is triangular in outline and contains dense cytoplasm, a large nucleus, and a darkly staining granule—the blepharoplast. The nucleus of the spermatid shifts toward the broader end, forms a beak, gradually increases in length, and eventually curves, becoming more or less signet-shaped (figs. 20, 21). The increase in length continues, and the nucleus becomes thinner and more coiled until finally, enveloped in a very thin cytoplasmic sheath, it forms the body of a spermatozoid. The relationship and

movements of the blepharoplast with respect to the nucleus have not been studied. A mature sperm shows two to three coils with the anterior end thin and tapering and the posterior thicker and strap-shaped. At the anterior end there are two cilia, while the posterior end bears a vesicle (fig. 22).

There are a number of chloroplasts in the wall cells of the fully developed antheridium. At maturity of the antheridium these chloroplasts turn brown and finally colorless.

The mature antheridium is borne on a stalk three to five cells long and two cells thick.

Female plant

The archegonia are produced in a cluster on the dorsal surface of the midrib just behind the growing point. As described by Kashyap, each cluster is surrounded by involucral bracts which may also be interspersed among the archegonia. Occasionally there may be two such clusters on a single plant, one located behind the other. The archegonial bracts are one layer of cells thick except at the base, where they may be two or three layers thick. The archegonia within a cluster may be at different stages of development as younger archegonia may arise among the older ones.

Development of archegonium.—The archegonia correspond closely in their development to those of the other anacrogynous Jungermanniales. Each archegonium arises from a single superficial cell in a manner similar to that of the antheridium. The papillate initial is divided by a transverse wall just above the general surface of the thallus, into an upper, slightly larger, archegonial initial and lower basal cell (fig. 23). The former stains more deeply than the latter. As is usual, the archegonial initial is divided by three vertical walls into three peripheral cells inclosing an axial cell (figs. 24–26). Simultaneously with these divisions of the archegonial initial the basal cell also divides to form two tiers of two cells each. In the archegonium the peripheral neck initials then divide by radial vertical walls forming five to eight cells surrounding the central one. Each of these peripheral cells forms a row of neck cells by transverse segmentation. Meanwhile the axial cell divides into an upper, smaller cover cell and a lower, larger one (fig. 27). The latter divides to form an upper primary neck canal cell and a lower central cell (fig. 28).

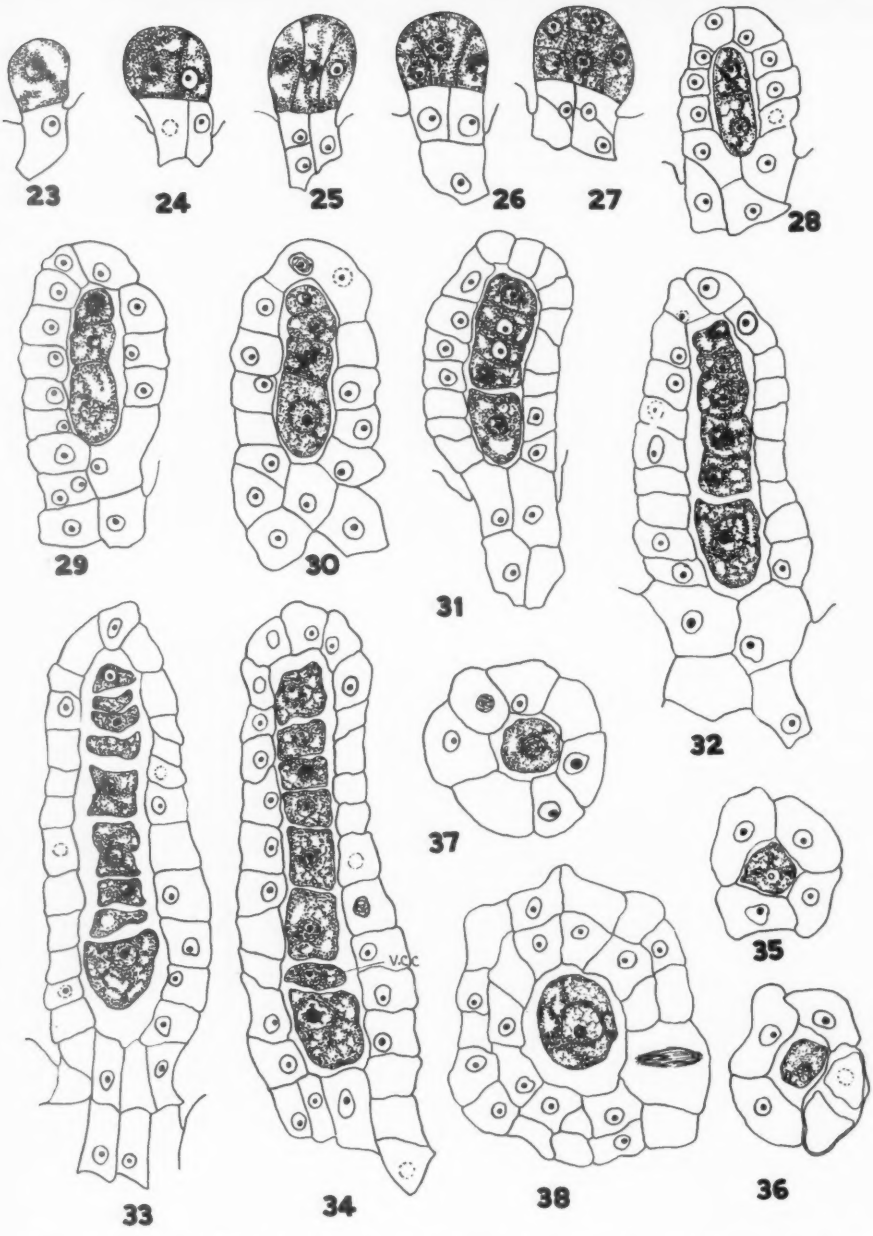
Two intersecting vertical walls divide the single cover cell into four cells which serve to close the neck of the archegonium.

The division of the primary neck canal cell invariably precedes that of the primary central cell. In a maturing archegonium six to nine neck canal cells, usually broader than they are long, are formed by successive transverse divisions (figs. 29–33).

The central cell divides at a later time into a ventral canal cell and the egg (fig. 34). The former gradually becomes compressed into a disk-shaped structure.

The neck of a fully mature archegonium is slightly curved and twisted as a result of unequal growth on the two sides. It is composed of five to eight rows of cells with eight to ten cells in each row. In one instance a transverse section showed four rows in the neck just below the cover cells (fig. 35), but a little lower down five rows of cells were found (fig. 36). The venter is at first only one cell in thickness (fig. 37) but becomes two- to four-layered after fertilization (fig. 38).

Development of perianth.—The development of the perianth has been described by Kashyap (2). Subsequent



FIGS. 23–38.—*Sewardiella tuberifera*. Figs. 23–32, stages in archegonial development. Figs. 33, 34, mature archegonia. Figs. 35, 36, transverse sections of archegonial neck at different levels. Figs. 37, 38, transverse sections of venter of archegonium; fig. 37, prior to fertilization; fig. 38, subsequent to fertilization.

to fertilization the involucral bracts surrounding the archegonial cluster are carried upward by zonal growth at the base, resulting in an inverted bell-shaped structure. Sometimes the growth is lacking at one or two places. In such cases the perianth cup shows one or two splits extending right to the base. Since some of the bracts are disposed toward the outside and others toward the inside of the ring of zonal growth and are carried upward in these positions, the perianth bears bracts on both the inner and the outer surfaces as well as on the margin. Strictly speaking, the bell-shaped structure is composite in nature as in *Petalophyllum indicum* (5). Often some of the bracts which lie in the center of the cup among the archegonia are left behind. Ordinarily the growth in size of the perianth bears a direct relation to the developmental stage of the sporogonium within, and one can easily predict the degree of development of the sporogonium from the size of the perianth.

Sporophyte

No stages of fertilization were observed. A number of archegonia may be fertilized in each group, but normally only one develops into a mature sporogonium. When two mature sporogonia were found on the same plant, they were occasionally within the same perianth but more frequently in separate perianths.

Early stages in embryo development were not secured. The earliest stage observed showed the laying-down of periclinal walls in the capsule region to cut off a single-layered amphithecium from the endothecium. The amphithecial cells divide further by both periclinal and anticlinal walls to give rise to the three- to four-layered wall of the capsule.

The spore mother cells and the elater mother cells belong to the same cell generation (figs. 39–41). The spore mother cells are characteristically four-lobed as in other members of the Jungermanniales and contain a large amount of food material.

By the time the sporogonium is at the mother cell stage, the foot is quite prominently developed. It is bulbous, of Douin's general type, with large cells sharply demarcated from the seta (fig. 46). The peripheral cells which are directly in contact with the tissue of the gametophyte are haustorial. The outer walls of these cells are papillose and thick (fig. 47), the cytoplasm dense, and the nuclei larger than in the other cells of the foot. The cytoplasm of the invaded gametophyte cells at first becomes frothy, and finally the cells perish, forming mucilaginous lamellations.

Cells of the young seta are more or less rectangular and highly meristematic.

Mature sporogonium.—The capsule becomes dark colored and rounded. Its wall is usually three or four layers of cells thick, and the cells of the outer layer are comparatively more elongated than those of the inner ones and have heavier outer walls. The cells of all layers show bands of thickening on the radial and inner walls, though these are less abundant in the cells of the outermost layer (fig. 45). These bands appear only with the maturation of the sporogonium and are not visible at the tetrad stage.

The seta is thick and short, so that the capsule barely emerges from the perianth. It is ten to fourteen cells across and is formed of rectangular cells (fig. 46).

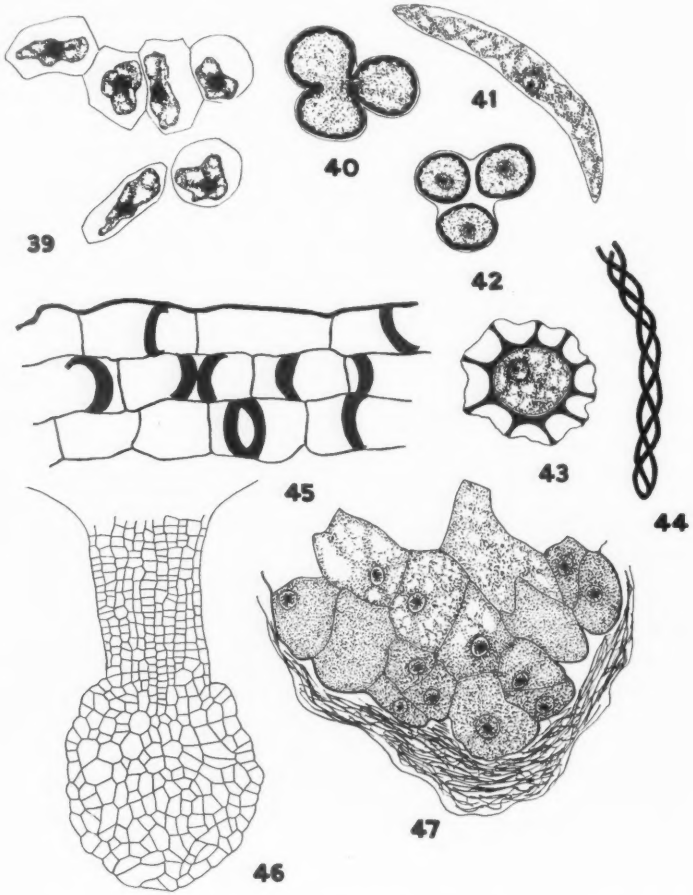
The mature spores are brown, reticulate lamellate and contain stored food (figs. 42, 43). The average diameter of the spores is 40–45 μ. The lamella of a spore is 6–8 μ thick. The elaters are 300–400 μ

long, bi- or trispiral and on the average 8 μ thick (fig. 44).

CHROMOSOME NUMBER.—The *2n* chromosome number for the species is reported to be eighteen (3). This is confirmed from sections of the capsule showing cells of the archesporium in a state of division. A dividing cell of the thallus shows the haploid number to be nine.

Discussion

The phylogenetic affinities of the genus and its place in the evolutionary series in the Fossombroniaceae have already been discussed by MEHRA and VASHISHT (5). These authors pointed out how the thallus of *Sewardiella* might have been derived from foliose forms like *Fossombronia* by the congenital concrescence of



FIGS. 39–47.—*Sewardiella tuberifera*. Fig. 39, archesporial cells. Figs. 40, 41, spore mother and elater mother cell, respectively. Fig. 42, tetrad in section. Fig. 43, mature spore. Fig. 44, part of elater showing three spiral thickenings. Fig. 45, transverse section through capsule wall. Fig. 46, bulbous foot and seta. Fig. 47, haustorial cells of foot.

dorsolateral leaves with an intermediate condition being represented by *Petalophyllum*.

A study of the apical cell further supports this view. In most acrogynous Jungermanniales the apical cell is tetrahedral and cuts off three series of segments, two dorsal and one ventral. The dorsal segments give rise to the dorsal rows of leaves and the ventral segments to the ventral row. Even though the thallose habit has been assumed, the ancestral type of apical cell has been retained in both *Petalophyllum* and *Sewardiella*. In both genera it is tetrahedral as found in the foliose forms.

Summary

1. The saddle-shaped vegetative body of *Sewardiella tuberifera* consists of a thick central midrib and two lateral thin flattened wings. Often the thallus is dichotomously branched.

2. The older ventral region of the midrib is profusely infected by an unseptate fungal mycelium which is probably in symbiotic relationship with its host. The fungus forms thick-walled vesicles which often fill the entire cavity of the infected cell.

3. The apical cell is tetrahedral with three cutting surfaces.

4. Perennation is accomplished by means of apical tubers.

5. The plants are strictly dioecious.

6. The antheridia are scattered on the dorsal surface of the midrib, each growing in the axil of a bract, though occasionally several may be present in the axil of a larger bract. They develop from the segments close to the apical cell and thus are strictly acropetal. Development is of the type usual in the Jungermanniales.

7. Archegonia of mixed ages are aggregated on the dorsal surface of the midrib into a cluster which is surrounded by bracts. Development follows the usual pattern in the Jungermanniales. The neck is composed of five to eight rows of cells, and as many as nine neck canal cells have been observed. The venter remains a single cell layer thick until fertilization.

8. The bell-shaped perianth develops by zonal growth at the base. The involucral bracts are carried up by this growth and not only form the lacerated margin of the cup but also occur on the inner and outer surfaces of the cup. Thus the perianth is of a composite nature.

9. Mother cells of the spores and elaters belong to the same cell generation. The protoplast of the spore mother cell is four-lobed, and tetrad formation following meiosis results from the deepening of the invaginations.

10. The bulbous foot is of Douin's generalized type, and its basal cells are haustorial in function. The capsule wall has three to four layers of cells, all of which show bands of thickening on the radial and inner walls. The spores are reticulate lamellate, and the small elaters are bi- or trispiral.

11. The haploid chromosome number is nine; the diploid, eighteen.

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ATYPICAL GROWTH OF PLANTS. III. GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX TO CERTAIN NUCLEIC ACID COMPONENTS AND RELATED COMPOUNDS[1]

LOUIS G. NICKELL, PHYLLIS GREENFIELD, AND PAUL R. BURKHOLDER

Introduction

The nucleic acids and their constituent purine and pyrimidine bases appear to have important roles in the metabolism, growth, and reproductive processes of all kinds of organisms. Recent researches have shown that polynucleotides exist in viruses (31) and play a vital part in the hereditary machinery of microbes and higher plants and animals (6, 33). As the chemistry of this class of compounds (19, 33–35) becomes better understood, progress is possible in determining the biological relationships of these substances. Thus, it has been shown that desoxyribosenucleic acid acts as a transforming principle in the conversion of one bacterial type into another (17), and it is now clear that the synthesis of proteins by living cells is coupled to the metabolism of nucleotides (4, 30). Since the demonstration by RICHARDSON (23) that hydrolytic products of nucleic acids may be growth factors for bacteria, numerous investigators have studied the action of purine and pyrimidine bases for bacteria (2, 5, 9, 13, 21, 26, 28, 29, 32), *Phycomyces* (24, 25), *Neurospora* (7, 16, 18, 22), *Tetrahymena* (10), and other organisms. In the voluminous literature of this field, one finds that many natural species and specially induced mutant strains of microörganisms require exogenous supplies of nucleic acid components.

While studying the reverse mutation of biochemically deficient strains of *Neurospora*, LEDERBERG (14) was led to develop a nutritional concept of cancer, which offers considerable promise for experimentation in the field of atypical growth. Autonomous and disorganized growth may be viewed as the end result of gene mutation (which might have occurred in a normal cell) which would then permit synthesis of a metabolite (vitamin, nucleic acid component, etc.), making possible relatively unrestricted biochemical activity which leads to atypical morphological expression. LEDERBERG pointed out, also, that the introduction of a virus into the system of protoplasm could provide the missing link for a blocked enzyme system and thus supply an alternative mechanism for gain in synthetic capacity. On this basis, tumor cells should differ in their growth-factor requirements from homologous normal cells when cultivated *in vitro*. Plant tumors and normal tissue should provide useful material for experimental tests of this hypothesis.

In the study of the physiology of

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higher plants, BONNER and HAAGEN-SMIT (1) have shown that adenine is a growth factor for leaf expansion, and it seems probable that this base may be limiting nucleotide synthesis in young growing leaves. More recently GALSTON and HAND (8) have demonstrated that adenine stimulates the growth of epicotyl sections, leaf buds, and roots of etiolated peas, as well as prevents, to a large extent, the thermal inactivation of growth of sections of excised pea epicotyl. Without going into an extensive and detailed review of the literature of biochemical genetics, nutrition, virology, etc., it is only suggested that the biological significance of nucleic acids and their constituents can scarcely be overestimated.

In recognizing the obvious importance of nucleic acid components for growth processes, we decided to investigate the responses of *Rumex* virus tumors to some of these substances, utilizing the advantages of a tissue-culture technique. Growth in relation to various purines and pyrimidines and one analogue of guanine in chemically defined media will be reported upon in this paper.

Material and methods

Tissue cultures of virus tumors of *Rumex acetosa* L. (sorrel) were employed in these studies, using the general methods reported earlier (3). These tumors carry the virus *Aureogenus magnivena* Black which causes the wound-tumor disease in certain plants. They exhibit the ability to grow well in sterile media containing relatively high concentrations of nitrate and phosphate. When grown on a favorable nutrient gel, in the presence of light, these tumors consist of loosely cohering meristematic nodules which are readily separated and capable of indefinite growth and which collectively form irregular light-green masses of tissue. Unfavorable conditions for growth, of any nature, result in a browning of the tissues which is proportional to the amount of growth inhibition (3).

Neither the nucleic acids nor any of their organic constituents were found necessary for good growth of the virus tumors in a chemically defined medium, but it seemed possible that the growth rate could be increased by the addition of such compounds as would be indicated on theoretical grounds to contribute toward the synthesis of virus and cell protein. The tumor tissue was the same strain used in our previous work (3, 20), and the basal medium contained the following: KNO_3, 0.002 *M*; $Ca(NO_3)_2$, 0.003 *M*; KH_2PO_4, 0.008 *M*; $MgSO_4$, 0.001 *M*; $CaCl_2$, 0.003 *M*; KCl, 0.002 *M*; $MgCl_2$, 0.001 *M*; sucrose, 2%; thiamin, 100 μgm/l; pyridoxin, 800 μgm/l; niacinamide, 800 μgm/l; Difco Noble washed agar, 1%; and trace elements added in p.p.m. as follows: B, 0.1; Mn, 0.1; Zn, 0.3; Cu, 0.1; Mo, 0.1; and Fe, 0.5. This medium was made according to directions given earlier, and the various compounds were added to it in concentrations as indicated in the individual experiments.

The method of expressing growth response is the same as that used before: "growth value" is the final wet weight divided by the initial weight of inoculum. Most of the experiments were run in replicates of five, but for certain compounds and concentrations sets of ten cultures were used. All the tumors were cultivated on agar slants for a period of 21 days.

Experimentation

INTACT NUCLEIC ACIDS.—In preliminary experiments, nucleic acids, obtained from commercial sources, were added in varying amounts to the basal

medium. Desoxyribosenucleic acid (DNA) inhibited growth and caused the tissue to become brown in color. Ribosenucleic acid (RNA) was somewhat inhibitory to growth but caused no striking visible changes. It was not clear whether the large molecules actually penetrated the cells, so it was decided to use component parts of the polynucleotides.

an alkaline hydrolysate of the two nucleic acids gave a curve (fig. 1) more or less parallel to that given by alkaline-hydrolyzed RNA, but somewhat lower.

The increase in growth value resulting from the addition of alkaline-hydrolyzed RNA and the decrease caused by the addition of hydrolyzed DNA leads to the postulation of at least two interpretations of the cause of this difference:

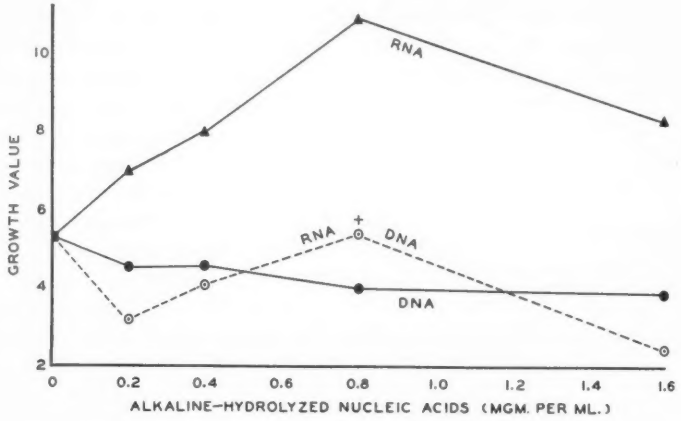

FIG. 1.—Growth responses of virus tumors to alkaline-hydrolyzed nucleic acids added to basal medium (no. 24). Growth value = final fresh weight/inoculum weight.

ALKALINE-HYDROLYZED NUCLEIC ACIDS.—Alkaline-hydrolyzed RNA, prepared according to LEVENE and BASS (15), was added in small amounts to the basal medium. Preliminary tests indicated the nucleotide mixture to be stimulatory, and, therefore, a dose-response experiment was conducted. The optimal concentration of alkaline-hydrolyzed RNA was 0.8 mg/ml of medium (fig. 1 and table 1). This experiment was followed by another using a concentration series of alkaline-hydrolyzed DNA. Results of this DNA experiment are also included in figure 1 and table 1 and show inhibition at all levels tested. The addition of equal amounts of

(*a*) Aside from the difference in the sugar components, these two nucleic acids, when completely hydrolyzed, show another important difference, i.e., the nature of one constituent pyrimidine (uracil in RNA, and thymine in DNA). It is therefore tempting to postulate that the stimulation of RNA results from its uracil (or uridine, uridylic acid) content and that the inhibition of DNA is related to its thymine (or thymidine, thymidylic acid) content. (*b*) The second hypothesis is that some other component, common to both acids, is inhibitory, while uracil is able to overcome that inhibition, and thymine is not.

NUCLEOTIDES.—Since the nucleotides

resulting from alkaline hydrolysis of RNA were stimulating when tested together, the individual nucleotides were tested to ascertain if the stimulation uracil is the only base in RNA which is not present in DNA.

An experiment needed to determine whether postulate (*a*) or (*b*) is correct

TABLE 1

GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX ON BASAL MEDIUM WITH ADDITION OF VARYING AMOUNTS OF ALKALINE-HYDROLYZED NUCLEIC ACIDS

MEDIA	AMOUNT NA ADDED (MG/ML)	RELATIVE COLOR*		GROWTH VALUE†
		Green	Brown	
Basal medium only	.....	6	0	5.26
Basal medium+RNA	0.2	6	0	7.00
" " +RNA	0.4	6	0	8.00
" " +RNA	0.8	6	0	10.88
" " +RNA	1.6	4	0	8.31
Basal medium+DNA	0.2	4	0	4.56
" " +DNA	0.4	4	0	4.70
" " +DNA	0.8	4	0	4.00
" " +DNA	1.6	4	1	3.83
Basal medium+DNA+RNA	0.2	4	0	3.04
" " +DNA+RNA	0.4	4	0	4.11
" " +DNA+RNA	0.8	5	0	5.33
" " +DNA+RNA	1.6	4	2	2.34

* Color of tumors is reported on an arbitrary scale increasing in intensity from 0 to 6.
† Growth value = Final fresh weight/inoculum weight.

TABLE 2

GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX ON BASAL MEDIUM WITH ADDITION OF NUCLEOTIDES

MEDIA	AMOUNT COMPOUND ADDED (MG/ML)	RELATIVE COLOR		GROWTH VALUE
		Green	Brown	
Basal medium only	.....	6	0	6.22
Basal medium+Adenylic acid	0.2	4	2	2.71
" " +Guanylic acid	.2	5	1	3.52
" " +Cytidylic acid	.2	5	1	4.74
" " +Uridine	0.3	6	0	8.00

resulted from any one alone. Because of the high costs and the scarcity of these compounds at the time, they were run at one level only (uridylic acid was unavailable and was substituted by uridine). From the results in table 2 it can be seen that only uridine was stimulatory. This result is according to expectation, since involved a concentration series of thymine. The results (fig. 2 and table 3) demonstrated that thymine has scarcely any effect on the growth of the tumor. These results suggested, therefore, that (*b*) is the correct interpretation, provided that some other component is inhibitory.

In order to test whether the limiting factor is the ability of the tumor tissue to link sugar to the base or to link phosphoric acid to the sugars, concentration adenosine-adenylic curves (fig. 2) provides some evidence necessary to show that (*b*) is more likely the answer to the inhibition-stimulation phenomenon

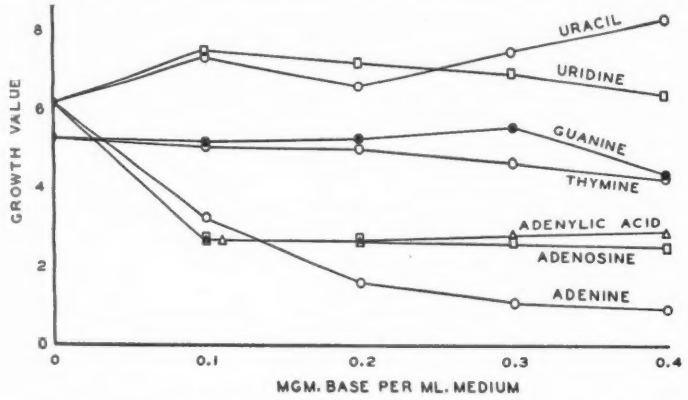

FIG. 2.—Growth responses of virus tumors to nucleic acid bases, their nucleosides, and nucleotides added to basal medium (no. 24). Nucleosides and nucleotides were added in equimolar amounts corresponding to their own base.

TABLE 3

GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX ON BASAL MEDIUM WITH ADDED THYMINE AND GUANINE

MEDIA	AMOUNT COMPOUND ADDED (MG/ML)	RELATIVE COLOR		GROWTH VALUE
		Green	Brown	
Basal medium only		6	0	5.30
Basal medium+Thymine	0.1	5	0	5.05
	.2	5	0	5.00
	.3	4	0	4.55
	0.4	4	0	4.22
Basal medium+Guanine–HCl	0.1	4	0	5.15
	.2	5	0	5.30
	.3	5	0	5.90
	0.4	4	1	4.18

series were set up with the adenine-adenosine-adenylic acid group and the uracil-uridine group. The resultant data (fig. 2 and table 4) show that, in all probability, the base itself may be the important structure, not either of the linkages.

The inhibition shown by the adenine-found in these studies. Experiments aimed at overcoming the inhibition by adenine through the addition of uracil to the medium have so far not yielded convincing results (fig. 3 and table 5). It is probable that other unrecognized factors are involved.

XANTHINE AND RELATED COMPOUNDS.

TABLE 4

GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX ON BASAL MEDIUM AND WITH INCREASING AMOUNTS OF ADDED COMPOUNDS AS SHOWN

Media	Amount compound added (mg/50 ml)	Relative color		Growth value
		Green	Brown	
Basal medium only		6	0	6.22
Basal medium+Adenine	5	5	1	3.26
	10	2	2	1.57
	15	1	3	1.11
	20	0	3	0.99
Basal medium+Adenosine	8.5	3	1	2.77
	17	3	1	2.72
	25.5	2	1	2.63
	34	2	2	2.54
	42.5	1	2	2.37
Basal medium+Adenylic acid	13	4	1	2.74
	15	4	1	2.71
	26	4	1	2.73
	39	4	1	2.77
	52	4	1	2.87
Basal medium+Uracil	5	6	0	7.36
	10	5	0	6.30
	15	5	0	7.51
	20	5	0	8.39
Basal medium+Uridine	11	6	0	7.51
	15	6	0	7.89
	22	5	0	7.24
	33	5	0	7.04
	44	5	0	6.11

—Because of the similarity between the xanthine–hypoxanthine–uric acid series and the nucleic acid bases, it was considered worth while to test the effect of a small amount of xanthine added to the basal medium. Growth of the tumors was stimulated, and a concentration series of these compounds was then prepared. At the concentrations used (0.1, 0.2, 0.3, and 0.4 mg/ml of medium), both xanthine and hypoxanthine were increasingly stimulatory (fig. 4 and table 6), the stimulation of hypoxanthine being somewhat higher than that of xanthine. Uric acid caused stimulation up to 0.2 mg/ml and inhibition above that level.

It should be mentioned here, also, that

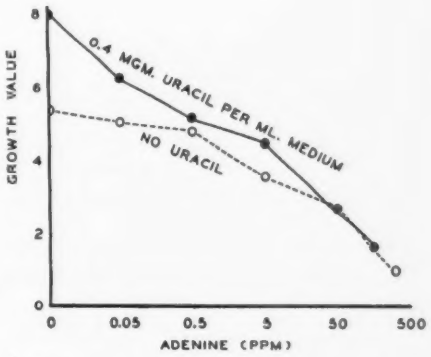

FIG. 3.—Effect of uracil in overcoming adenine inhibition of growth of virus tumors.

in later experiments one sample of the base guanine (Schwartz) added at a level of 0.3 mg/ml raised the growth value from a basal level of 8.8 to 10.6. Another specimen of guanine hydrochloride (Eastman), used at this same level, effected a decrease of the growth value below that of the controls.

TABLE 5

GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX ON BASAL MEDIUM WITH AND WITHOUT URACIL AND WITH VARYING AMOUNTS OF ADENINE

Adenine added (p.p.m.)	Relative color		Growth value
	Green	Brown	
	No uracil added		
0	5	0	5.36
200	1	2	1.00
100	2	1	1.75
50	3	1	2.80
5	4	1	3.60
0.5	5	0	4.83
0.05	5	0	5.13
	Uracil added (0.4 mg/ml)		
100	2	2	1.70
50	3	1	2.70
5	4	0	4.53
0.5	5	0	5.18
0.05	5	0	6.25
0	6	0	8.00

In view of the results obtained with various purines and pyrimidines, it is interesting that all those bases which stimulate growth (guanine, hypoxanthine, xanthine, uric acid, and uracil) possess a keto group in the number 6 position.

INHIBITION BY GUANAZOLO.—In view of the reported inhibition of growth by guanazolo (12) supplied to *Tetrahymena* and to malignant mouse tissue, we decided to test the effect of this substituted purine on the growth of the virus tumor. Through the kindness of Dr. G. W. KIDDER an adequate amount of the compound was made available. In preliminary tests it was found, in concentrations as low as 0.001–0.05 mg/ml of medium, to inhibit the growth of virus tumor and also tissue from *Nicotiana tabacum* cultured *in vitro* and the *tabacum* tissue containing tobacco mosaic virus. Tests were then made to determine whether the addition of guanine to a medium containing small amounts of guanazolo would overcome the inhibition; the results indicated antagonism between these two structurally related compounds, as had already been demonstrated in other organisms (11). A final experiment was set up to show the release from guanazolo inhibition by increasing doses of the base guanine. The resulting data are presented in figure 5 and table 7.

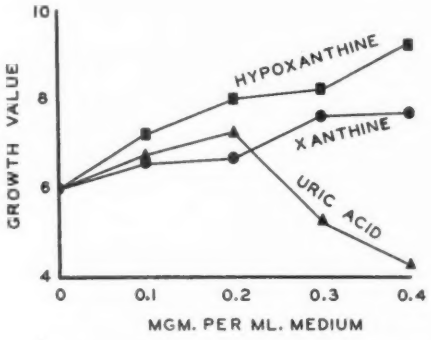

FIG. 4.—Growth responses of virus tumors to hypoxanthine, xanthine, and uric acid added to basal medium (no. 24).

Control tumors grown on basal nutrient agar yielded a growth value of 8.85. Addition of guanine at the level of 0.3 mg/ml raised this value to 10.64. In the presence of guanazolo, added at 0.001 mg/ml, the growth value was lowered to 3.83. When the medium contain-

TABLE 6

GROWTH RESPONSES OF VIRUS TUMORS OF RUMEX ON BASAL MEDIUM AND WITH ADDITION OF HYPOXANTHINE, XANTHINE, OR URIC ACID

MEDIA	AMOUNT COMPOUND ADDED (MG/50 ML)	RELATIVE COLOR		GROWTH VALUE
		Green	Brown	
Basal medium		6	0	6.00
Basal medium+Xanthine	5	5	0	6.60
	10	5	0	6.71
	15	5	0	7.04
	20	5	0	7.70
Basal medium+Hypoxanthine	5	5	0	7.24
	10	5	0	8.05
	15	5	0	8.25
	20	5	0	9.25
Basal medium+Uric acid	5	5	0	6.72
	10	6	1	7.30
	15	5	1	5.32
	20	5	2	4.30

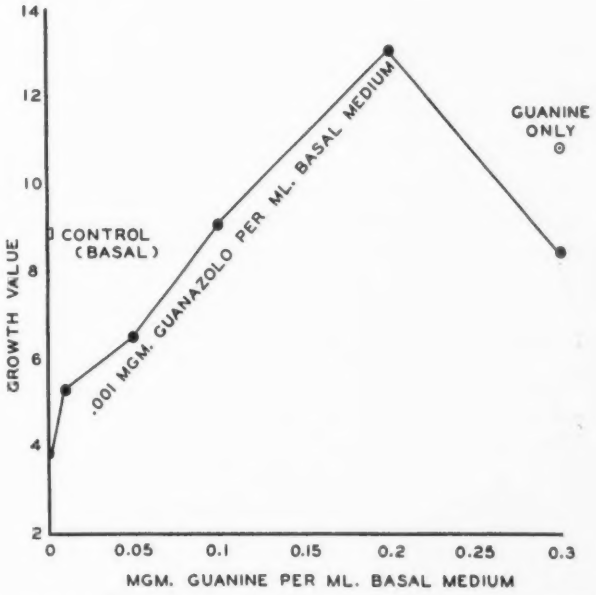

FIG. 5.—Ability of guanine to overcome inhibition by guanazolo of growth of virus tumors

ing guanazolo at 0.001 mg/ml was supplemented with increasing doses of guanine from 0.01 to 0.20 mg/ml, the growth value rose steadily to 13.15. When the ratio of guanine to guanazolo was in the region of 100:1 or 200:1, the inhibitory action of guanazolo was blocked, and excellent growth resulted.

Discussion

Although several purines and pyrimidines are required for growth of numerous microörganisms, these compounds have not been recognized as vitamins for higher organisms. The promotion of leaf growth by adenine, found by BONNER and HAAGEN-SMIT (1) in their studies on growth factors of higher plants, and the stimulation by the same compound of growth of epicotyl sections, leaf buds, and roots of etiolated peas found by GALSTON and HAND (8), appear to stand alone as examples of purine stimulation in the physiology of higher plants. The importance of adenine in the control of differentiation of tobacco stem segments and tobacco callus developed from these segments has been shown recently by SKOOG and TSUI (27). Our observations on the inhibitory influence of adenine on the growth of virus tumor derived from the root of *Rumex* provides a contrast of some interest. It remains to be determined whether adenine, adenosine, and adenylic acid have peculiar inhibitory properties for this tumor. The growth-promoting properties of the ketopurines, guanine (Schwartz), xanthine, and hypoxanthine, and the ketopyrimidine uracil, provide another example of growth-factor activity in a special kind of cellular metabolism, i.e., a plant tumor containing the causative virus.

TABLE 7

INHIBITION OF GROWTH OF VIRUS TUMORS BY GUANAZOLO AND RELEASE OF INHIBITION BY GUANINE

Guanazolo (mg/ml)	Guanine (mg/ml)	Initial weight (mg.)	Final weight (mg.)	Growth value
0	0	140	1240	8.85
0	0.3	125	1330	10.64
0.001	.0	120	460	3.83
.001	.01	125	655	5.24
.001	.05	110	720	6.55
.001	.10	130	945	9.09
.001	.20	135	1775	13.15
0.001	0.30	120	1015	8.46

The striking difference between the effects of alkaline-hydrolyzed DNA and RNA apparently cannot be explained solely by adenine inhibition released by the uracil present in RNA. It is possible that certain proportions of several constituents of RNA are required to bring about a physiological balance and thus protect against the toxicity of the adenine compounds (fig. 2). It is also possible that other compounds, not ordinarily considered components of the nucleic acids, may play a part in the overcoming of adenine toxicity (fig. 4).

At the higher levels of ketopurines employed (0.3 and 0.4 mg/ml), it is interesting that the beneficial effect upon growth of the tumors decreases with an increase in the number of keto groups (fig. 4). The 6-ketopurine (hypoxanthine) is stimulatory; 2,6-diketopurine (xanthine) is less beneficial; and 2,6,8-triketopurine (uric acid) is definitely toxic at the higher levels used.

The striking inhibition of growth exhibited by the triazolo analogue of guanine and its complete release by guanine is in general agreement with the observations of KIDDER and DEWEY (11) on the protozoan *Tetrahymena geleii*. The sensitivity of tumor tissue to guanazolo is of interest in itself, but the ratio of approximately one hundred guanine molecules required to reverse one molecule of the

triazolo differs from the usual inhibition ratios. Apparently the enzyme system whose role in our experimental tissue is to catalyze the formation of guanine nucleoside and nucleotide has a very great affinity for the triazolo form. There would result then unnatural complexes which would lack the properties required to produce functional nucleic acid in the cells. It should be profitable to carry these studies further by extending the kinds of compounds and making a comparison of their effects upon growth of the tumor and of the normal tissue of *Rumex*.

Summary

1. Virus tumors of *Rumex acetosa* L. (sorrel) containing the virus *Aureogenus magnivena* Black were grown in culture in a chemically defined medium with various additions of nucleic acids, or their components, and related compounds.

2. Growth was enhanced by the addition of alkaline-hydrolyzed ribosenucleic acid in the range of 0.2–0.8 mg/ml and decreased by comparable levels of alkaline-hydrolyzed desoxyribosenucleic acid.

3. Marked inhibition was observed in media containing adenine, adenosine, or adenylic acid.

4. Some evidence of stimulation was observed in media containing guanine, uracil, xanthine, or hypoxanthine. At concentrations of 0.3 or 0.4 mg/ml of medium, 6-ketopurine (hypoxanthine) was stimulatory; 2,6-diketopurine (xanthine) less beneficial; and 2,6,8-triketopurine (uric acid) definitely toxic.

5. The triazolo analogue of guanine was inhibitory at all levels tested down to 0.001 mg/ml. This inhibition was progressively reversed by guanine added over a range from 0.01 to 0.30 mg/ml.

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INHIBITION OF GROWTH IN MAIZE EMBRYOS BY CANAVANINE AND ITS REVERSAL[1]

JAMES E. WRIGHT[2] AND ADRIAN M. SRB

Introduction

The natural amino acid canavanine, a structural analogue of arginine, is known to be a strong inhibitor of growth in *Neurospora crassa* (8) and in certain bacteria (12). More recently, BONNER (1) has shown that this substance is highly inhibitory to indoleacetic acid–induced elongation of *Avena* coleoptile sections. The addition of appropriate amounts of arginine results in the alleviation of inhibition by canavanine in all these organisms. These amino acids, then, may be considered to be metabolic antagonists, and there is fairly good evidence that their relationship, at least in *Neurospora* and some of the bacteria, is competitive.

Although not so effective as arginine,

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lysine and, to a lesser degree, methionine tend to reverse canavanine inhibition of *Neurospora* growth. Among the bacteria studied by VOLCANI and SNELL (**12**) growth inhibition by canavanine could be partially relieved by ornithine or citrulline in organisms able to utilize these last-named amino acids for conversion into arginine. For *Avena* coleoptiles both glutamic acid and lysine, as well as arginine, show appreciable activity in opposing the effects of canavanine.

The present paper describes effects of canavanine on growth of excised, immature maize embryos cultured *in vitro*. Evidence is presented which shows that canavanine inhibits the growth of such embryos and that this inhibition can be reversed by the addition of arginine or certain other amino acids. Some data are also reported which indicate differential effects of canavanine on maize embryos of genetically diverse origin.

Material and methods

The maize inbred lines used in this study were grown in the greenhouse during the winter of 1948–49. They were self- or cross-pollinated in April, and the embryos were excised 18–40 days after pollination. Because the pistillate inflorescence emerged several days after the anthers had begun to dehisce in many of the inbreds, it was impossible to self some of them, and few reciprocal crosses could be made.

The method of excising and culturing embryos was adapted from that of HAAGEN-SMIT *et al.* (**6**). At the desired age, ears were husked, dipped into 70% ethanol, and rinsed with sterile distilled water. The tops of the kernels were then sliced off with a sterile scalpel, the depth of the slice depending upon the size of the kernel and embryo. Each embryo was teased out with a dissecting needle and placed on the surface of artificial culture medium contained in 18 × 150-mm. test tubes. To prevent contamination from air-borne spores, this work was done in a portable airtight chamber which was sprayed down before each operation with 1% phenol solution. Among over a thousand cultures handled in this way, no contamination was observed.

The basic artificial medium used was that described by TUKEY (**11**), to which was added agar and sucrose at concentrations of 0.65% and 3%, respectively. Ten ml. of this medium plus desired supplements were distributed into each test tube; the tubes were then fitted with cotton plugs and autoclaved at 10 pounds pressure for 20 minutes. After embryos were transferred to these tubes, they were incubated at 30° C. in a dark chamber for 10 or 15 days. The cultures were then taken down, the seedlings washed in warm distilled water to remove adhering medium, placed in envelopes, and dried at 80° C. for 48 hours. The scutellum was removed from the seedlings before individual dry weights were taken on an analytical balance.

The canavanine used was isolated from jack-bean meal and obtained in its final form as the sulfate. The method was one suggested by HOROWITZ and FLING (**7**) as a modification of the procedures of GULLAND and MORRIS (**5**) and of CADDEN (**3**).

Results

In a preliminary experiment using 24-day-old embryos of Inbred NY 1, it was found that canavanine added at the rate of 20 mg/l effectively inhibited growth. The plumules and radicles of the embryos elongated to approximately 5 mm. in length and then ceased growth, becoming discolored by the tenth day in culture. This inhibition by canavanine did

not occur in those cultures to which arginine was added in a concentration of $M/5000$. On the basis of these results, a series of experiments was set up to determine the reaction to canavanine of genetically different maize inbred and hybrid embryos excised at different ages. Representative data are shown in table 1. experiments of an exploratory nature, mature whole kernels of WF 9 and WF 9 × 38-11 were surface sterilized with a 2% solution of calcium hypochlorite and placed on each of the three media listed in table 1. Within 5 days all seedlings had grown completely out of the test tubes, with no apparent inhibition in

TABLE 1

INHIBITION OF GROWTH OF MAIZE INBRED AND HYBRID EMBRYOS BY CANAVANINE AND REVERSAL OF THE INHIBITION BY ARGININE

MAIZE INBREDS OR HYBRIDS	AGE IN DAYS OF EMBRYO WHEN EXCISED	NO. OF DAYS IN CULTURE	NO. OF REPLICATIONS	MEAN DRY WEIGHTS OF SEEDLINGS (MG.)			
				Control (No supplement)	Control + canavanine (20 mg/l)	Control + canavanine (20 mg/l) + arginine ($M/5000$)	% reversal
38-11 ⊗	18	10	5	6.2±0.4	0+	3.0±0.6	48.4
38-11×K 64	18	10	5	7.3±1.1	0++	6.2±0.8	84.9
K 64 ⊗	18	10	5	6.3±1.8	0+	4.8±1.8	76.2
K 64×38-11	18	10	5	10.4±3.2	0++	6.0±1.5	57.7
38-11 ⊗	19	10	4	9.0±0.2	0.4±0.2	4.6±0.9	51.1
38-11×WF 9	19	10	4	14.6±1.8	1.2±0.3	8.3±2.9	56.8
38-11 ⊗	21	10	4	14.0±2.4	1.2±0.2	7.6±0.8	54.3
CI 21×R 20*	21	15	4	22.0±2.7	1.4±0.2	12.6±1.5	57.3
K 64 ⊗	22	15	4	12.1±3.1	0.6±0.1	9.0±2.4	74.4
NY 1 ⊗	24	10	10	17.6±1.7	2.0±0.2	19.2±2.6	100.0
38-11×CI 21	24	15	4	22.8±5.0	1.9±0.4	21.0±4.0	92.1
Kys (open)	30	10	6	13.5±2.0	0.9±0.2	4.3±0.6	31.8
Kys ⊗	35	10	6	24.1±2.7	5.0±0.7	12.7±1.3	52.7
WF 9×38-11	40	10	6	41.3±4.5	15.7±1.7	24.2±3.5	58.6

* Pollen obtained from a Guatemalan open-pollinated variety grown by Dr. L. F. Randolph.

Inhibition was obtained in all lines tested. In some cases there is indication that hybrids are less sensitive to canavanine than are their component inbreds and that in the hybrids reversal of the inhibition by adding arginine is more complete. The data, however, are not sufficiently complete to permit generalization on this point. It is at least clear that genetically different strains show variation in their sensitivity to canavanine. There is also some evidence that age of the embryo when excised is a factor in sensitivity. In this connection, in those cultures grown in the presence of canavanine.

Table 2 reports the effects of various levels of concentration of canavanine and canavanine plus arginine on the growth of 21-day-old embryos of Inbred 38-11. It will be noted that the degree of inhibition increases as the canavanine concentration increases; within limits, at a given concentration of canavanine, alleviation of the inhibition increases with arginine concentration. The results show, however, that an arginine concentration of 160 mg/l is not so effective as

one of 80 mg/l in antagonizing the effects of canavanine. This is somewhat surprising inasmuch as the higher concentration of arginine does not seem to inhibit embryos grown in the absence of canavanine.

In another series of experiments, various amino acids were tested for their capacity to relieve inhibition by canavanine. These were added at final concentrations of $M/5000$ and $M/2500$ to medium containing 20 mg. of canavanine per liter. Embryos of two hybrids, 38-11 × CI 21 at 24 days of age, and CI 21 × R 20 at 21 days of age, were used. The results are presented in table 3.

Citrulline, ornithine, glutamic acid, lysine, and possibly methionine, besides

TABLE 2

GROWTH IN 10 DAYS BY EMBRYOS OF INBRED STRAIN 38-11 EXCISED 21 DAYS AFTER POLLINATION
VALUES REPRESENT MEAN DRY WEIGHTS OF SEEDLINGS IN MG.
BASED ON FOUR REPLICATIONS

CANAVANINE IN MG/L	ARGININE IN MG/L					
	160	80	40	20	10	0
80	5.0±0.7	4.6±0.4	2.4±0.4	1.6±0.4	1.1±0.2	<0.1
40	6.0±0.5	8.1±1.5	4.3±1.2	2.6±0.5	2.1±0.3	0.8±0.2
20	7.6±1.2	10.5±1.2	7.6±0.8	6.5±0.4	5.1±0.2	1.2±0.2
10	8.1±1.8	13.6±2.1	13.4±3.1	9.5±2.1	4.2±0.6	1.3±0.5
0	14.5±3.6	15.2±3.4	14.5±1.7	16.4±2.3	15.0±2.2	14.0±2.4

TABLE 3

EFFECT OF AMINO ACIDS ON CANAVANINE INHIBITION OF MAIZE EMBRYOS. EMBRYOS OF STRAIN 38-11×CI 21 WERE EXCISED 24 DAYS AFTER POLLINATION; THOSE OF CI 21×R 20 AT 21 DAYS OF AGE. VALUES REPRESENT MEAN DRY WEIGHTS IN MG. OF GROWTH OF SEEDLINGS IN 15 DAYS, BASED ON FOUR REPLICATIONS

Supplement	Concentration	Growth of 38-11×CI 21	% of control	Growth of CI 21×R 20	% of control
None (control)		22.8±5.0		22.0±2.7	
Canavanine	20 mg/l*	1.9±0.4	8.3	1.4±0.2	6.4
Canavanine+arginine	$M/5000$	21.0±4.0	92.1	13.8±1.5	62.7
	$M/2500$			15.8±1.2	71.8
Canavanine+lysine	$M/5000$	8.0±1.9	35.1	2.4±0.4	10.9
	$M/2500$	11.3±1.9	49.6	1.8±0.1	8.2
Canavanine+glutamic acid	$M/5000$	8.8±1.2	38.6	10.2±1.7	46.4
	$M/2500$	17.0±1.8	74.6	12.9±1.3	58.6
Canavanine+citrulline	$M/5000$	12.3±1.7	53.9	8.7±2.6	39.5
	$M/2500$	13.5±1.6	59.2	10.7±0.7	48.6
Canavanine+ornithine	$M/5000$	10.8±0.8	47.4	10.5±1.9	47.7
	$M/2500$	15.4±1.2	67.5	13.2±1.9	60.0
Canavanine+ornithine and citrulline	$M/10000$+ $M/10000$			12.6±1.7	57.3
	$M/5000$+ $M/5000$	13.2±0.9	57.9	14.8±3.0	67.3
Canavanine+histidine	$M/5000$	2.6±0.5	11.4	1.9±0.3	8.6
	$M/2500$	3.6±0.8	15.8	1.4±0.3	6.4
Canavanine+methionine	$M/5000$	4.8±0.3	21.0		
	$M/2500$	5.2±1.2	22.8		

* Concentration of canavanine in all cultures except control.

arginine, are effective in counteracting canavanine inhibition of growth of these maize embryos. Other amino acids tested were without appreciable effect. These included leucine, isoleucine, valine, threonine, tyrosine, proline, and glycine, whose effects were very similar to those of histidine, which is included in table 3 for comparison. It is interesting that lysine was effective with one hybrid but not with the other.

Experiments were conducted to determine the effect of light, temperature, pH, and sucrose concentration of the medium on the inhibition. None of these factors significantly altered the response of 22-day-old embryos of Inbred K 64 to the inhibition or to its reversal by arginine.

Discussion

From these results it seems likely that canavanine acts as a metabolite antagonist of arginine in maize, as has been a suggested mechanism for the similar inhibition in other organisms (**1, 8, 12**). The partial reversal of the inhibition by ornithine and by citrulline might lead to the interpretation that these compounds are precursors of arginine in maize as well as in the mammalian liver (**9**), in *Neurospora* (**10**), and in other organisms. As pointed out by BONNER (**1**) in connection with work on *Avena* coleoptiles, the effectiveness of glutamic acid in relieving canavanine inhibition may be related to the fact that glutamic acid seems to be a precursor in the biosynthesis of arginine in certain microörganisms. In this connection it may also be worth recalling that BORSOOK and DUBNOFF (**2**) and COHEN and HAYANO (**4**) have shown that glutamic acid takes an active part in the conversion of citrulline to arginine by slices of guinea-pig kidney and by homogenates of rat liver, respectively. The effect of lysine in relieving canavanine inhibition of maize growth parallels results already obtained in *Neurospora* and *Avena*. The basis of this effect is still entirely obscure.

Summary

1. The growth of maize embryos cultured *in vitro* is strongly inhibited by canavanine.

2. This inhibition may be counteracted by addition of arginine to the culture medium.

3. Citrulline, ornithine, glutamic acid, and lysine are also markedly effective in relieving the inhibitory effects of canavanine.

4. Different genetic lines of maize seem to show variation in their sensitivity to canavanine inhibition and to the reversal of this inhibition by other amino acids.

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EFFECTS OF EXPOSURES OF SEEDS TO VARIOUS PHYSICAL AGENTS
I. EFFECTS OF BRIEF EXPOSURES TO HEAT AND COLD ON GERMINATION AND LIGHT-SENSITIVITY[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **615**

S. M. SIEGEL

Introduction

In the field of temperature relations of plants those aspects dealing with brief treatments of nondormant seeds (i.e., seeds able to germinate promptly on maturity) with heat or cold have hitherto been little investigated. The main work in this area has centered around studies involving death from heat and cold; these were reviewed by BELEHRADEK (**2**) and HEILBRUNN (**6**). CROCKER (**3, 4**) pointed out that the responses of seeds to light during germination may be altered by employing different incubation temperatures or alternating temperature conditions. He, however, dealt principally with species in which light is a factor in dormancy. He also discussed chemical and mechanical treatments in relation to dormancy and light-sensitivity and concluded that small grains, many legumes, and certain other groups of species are not, in general, light-sensitive. CROCKER made the general statement that seeds capable of rapid germination are not light-sensitive.

The sensitivity of seeds to light is considered in the present paper primarily as it is affected by temperature. Although CROCKER (**3, 4**) reviewed some work on this topic and TOOLE and TOOLE (**11**) made specific mention of crabgrass—one of the species considered in this paper—the ease with which the responses of some species to light may be altered by brief temperature treatments is apparently reported for the first time in the present work.

The treatments described extended from 20 to 60 minutes, in general, at various high or low temperatures. Effects of such treatments on germination, light-sensitivity, and, to a limited degree, on certain enzymes are described.

It must be emphasized that the data presented are preliminary; the object of these first experiments was to determine if the treatments employed were capable of having any effect whatsoever on the experimental materials. Although sufficient statistical numbers of seeds were used, the responses tabulated are those for the particular seed lots tested and do not necessarily apply to all possible seed populations of a given species. Such factors as age, variation in genetic composition, previous physiologic history, and geographic origin alter the final results. In addition, the data on germination are not, in themselves, sufficient for the drawing of many general conclusions.

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The writer has found that the growth responses are of equal, if not greater, importance in judging the nature of the effects which the treatments have on the seed. Work in the process of completion indicates that a variety of growth responses, such as stunting, may be evident for periods of several months following such treatments.

Material and methods

Tall crabgrass, *Digitaria sanguinalis* (L.) Scop.; alkali sacaton, *Sporobolus airoides* (Torr.) Torr.; and evening primrose, *Oenothera biennis* L., were the wild species employed. The grains of crabgrass were collected near Ames, Iowa, in March, 1947, those of alkali sacaton in 1943, and the seeds of evening primrose in the autumn of 1948 in the Chicago region. Seeds of cultivated species were those of *Triticum vulgare*, *Linum usitatissimum*, *Phaseolus vulgaris*, *Raphanus sativus*, and *Zea mays*, obtained from supplies purchased from commercial sources; all were of the 1947 crop, except flax and corn, both of the 1945 crop. These seeds were employed in experiments carried out between July, 1948, and May, 1949. All seeds were stored at 20°–25° C. for at least 1 month prior to use and in most cases for the greater part of the period from collection to use.

Seeds were germinated on moist filter paper in 9-cm. Petri dishes. Either three replicates of fifty to one hundred seeds or two replicates of one hundred seeds were used for each species at each treatment. In the course of preliminary work it was found that emergence of the hypocotyl or primary root is a more reliable criterion of successful germination than is the development of the aerial portion of the seedling. This is in agreement with the findings of Fox (5). In order to determine which of those seeds counted as germinated could have become established as seedlings, plants were grown for 3–7 days after germination was recorded. In almost all cases reported here there was found a direct relationship between germinability as counted and subsequent viability of the seedling.

Sterile conditions were maintained in the dishes. The oily or proteinaceous seeds (flax, bean, etc.) were sterilized by soaking in 0.5% Semesan solution; the grains of crabgrass and sacaton were too small to be easily treated but were found to be highly resistant to infection, thus requiring less aseptic conditions for successful germination.

The method of the experiment may be divided into two parts, the conditioning phase and the incubation phase. The former consisted of the exposure of separate groups of seeds containing different amounts of water to one or another of several temperatures. The required initial imbibition was carried out in a room with a light intensity of no more than 20 foot-candles at a room temperature of 23°–25° C., after which the seeds were immediately placed in the conditioning temperatures. Several durations of these conditioning temperatures were employed, but for the most part only those of 60-minute duration are here reported upon. In many cases the data on effect of duration of conditioning do not lend themselves to simple interpretation. Further experimentation and study are required before accurate time-temperature relationships in such conditioning can be established.

With the termination of the temperature conditioning the incubation phase began. Every temperature-conditioned group consisted of a pair of Petri dishes or a paired set of dishes consisting of duplicates or replicates. One half of each

group or pair was transferred to an incubation room which was dark; one half, to a room in light. The temperatures of these rooms were continuously recorded and were found to be almost identical with those in the Petri dishes. The maximum light intensities incident on the seeds during the incubation period were measured with a Weston photoelectric light meter. At the end of 70–90 hours of incubation, germination counts were made.

As an illustration of the conditioning process, data for crabgrass reported here cover grain populations with four different water contents: 9 (air-dry), 27, 39, and 44% (fully imbibed). These values were chosen after an imbibition curve for the grains had been determined. Groups with each of these water contents were each divided into six subgroups, five of these being conditioned in the dark for 1 hour with an air temperature of 10°, 40°, 50°, 60°, or 75° C.; air temperatures were maintained within 1° C. of those specified within a refrigerator or constant-temperature ovens. Another group, the control, was maintained at a room temperature of about 25° C.

Each temperature-conditioned group consisted of a pair of Petri dishes each containing three replicates of a hundred grains. Following 1 hour of temperature conditioning, one of each pair of dishes was transferred to light, the other to darkness; this initiated the incubation period. The incubation temperature involved the normal diurnal cycle for early August with a mean maximum of about 31° C. occurring between 11:30 A.M. and 1:00 P.M. and a mean low temperature of 15° C. occurring between 4:00 and 5:00 A.M. for the 3-day period. The temperatures listed above were the air temperatures in the incubation rooms. The groups in darkness were maintained in darkness constantly; those in light were illuminated by sunlight falling on the greenhouse roof for about 12 hours out of every 24. The maximum light intensity incident upon the seeds, about 3000–5000 foot-candles, occurred about the same time of day as the maximum temperature. The experiment on crabgrass was begun on August 7, 1948, and terminated on August 15, 1948. Germination counts, however, were made 72 hours after the experiment was begun. Representatives of some groups were planted or otherwise continued for some days after germination percentages were recorded.

Sacaton, radish, and flax were all handled in a manner essentially similar to that with crabgrass in experiments carried out from December, 1948, to May, 1949. Temperature conditions during incubation were maintained at about the same values as those in the experiment with crabgrass, but the maximum light intensity in the dishes was only about 250–350 foot-candles. This renders invalid a comparison of data from the crabgrass experiment with those for these species.

In a set of experiments with flax, corn, radish, and bean, carried out in March, 1949, the effect on germination of exposing the seeds to temperatures of approximately 100° C. for periods of 20, 40, or 60–70 minutes was determined. Seeds with several water contents were employed, but only the results obtained with dry seeds will be considered. Again, germination was carried out in both light and darkness. Corn and flax were comparable with each other with respect to age of seed, as were radish and bean.

Within a given lot of seeds, if age, origin, previous storage conditions, and other factors were the same, reproducibility of data was excellent. Some investi-

gations by the author have indicated that different lots of seed having different histories do not necessarily respond in the same manner to a given treatment.

The data were analyzed statistically by means of the "analysis for variance" (9). Particular points of interest were also compared by means of the "standard error of a ratio." The term "significant," as here used, means a statistical significance at most at the 5% level.

Results

Crabgrass

Crabgrass grains conditioned when air-dry (9% water content) and incubated in light showed a higher percentage of germination with the more elevated conditioning temperatures between 10° and 60° C., while those incubated in darkness showed lower percentages of germination after conditioning at these same temperatures (table 1). Both of these relationships are highly significant. Germination was less with conditioning at 75° than at 60° C. for groups in both light and darkness; differences are obviously of a high order of magnitude. It is of interest that three general responses may be distinguished when germination in light and darkness are compared. Grains conditioned at 10° C. germinated significantly more in darkness than in light; grains preheated at 50° C. or more showed higher percentages of germination in light than in darkness. When grains were conditioned between these temperatures (10°–50° C.), germination was essentially the same in light and darkness. It is noteworthy that after the 75° C. conditioning, although no germination was observed in darkness, 28% of the grains germinated in light. When the dark-incubated grains conditioned at 75° C. were transferred to light, after 7–10 days in the dark, germination of about 10–30% was recorded. After longer periods (up to 25 days) in darkness grains so treated did not germinate on being transferred to light. The failure to germinate in darkness may be considered an indication of light-obligate condition in the grains. The failure of grains incubated in the dark for periods of more than 10 days to germinate in light is similar to the so-called "dunkelhart" (darkhard) condition described for certain light-sensitive species (3, 4). If grains which had received conditioning at 75° C. when dry were incubated in darkness and 3–4 ml. of $M/100$ glucose per Petri dish were added, 2–5% germination occurred within a week; addition of glucose had no effect on those grains germinating in light.

TABLE 1

PERCENTAGE GERMINATION OF CRABGRASS UNDER VARIOUS CONDITIONS

Water content of grain when conditioned	Incubated in	Conditioning temperature (° C.)					
		10	25*	40	50	60	75
9%	Light	67	70	70	75	79	28
	Darkness	79	68	67	65	61	0
27%	Light	71	70	70	65	49	0
	Darkness	76	70	69	67	36	0
39%	Light	78	66	67	62	2	0
	Darkness	84	67	74	69	0	0
44%	Light	80	73	81	85	4	0
	Darkness	85	72	80	77	0	0

* Controls.

Grains conditioned with a water content of 27% showed progressively lower percentages of germination in both light and darkness with successively higher conditioning temperatures over the interval 10°–50° C. The lower percentages of germination in darkness after exposure

to 50° C. is significant. With this higher water content the conditioning temperature which reduced germination markedly was shifted from 75° to 60° C.; there was no germination in grains conditioned at 75° C. As was the case with the air-dry grains, after conditioning at 60° C. germination was greater in light than in darkness.

The grains with a water content of 39% were nearly fully imbibed when conditioned but, having been on a moist substrate for only 3 hours, were not yet at a high level of metabolic activity. Their percentages of germination in light were progressively less after conditioning at higher temperatures between 10° and 50° C; germination was considerably less after conditioning at 60° C. than after exposure to lower temperatures. In darkness, germination was obviously greater after conditioning at 10° or 40° C. than in the controls. In this latter comparison the difference in percentage of germination between either of these conditioned groups and their control was highly significant (odds over 99 to 1); this was on the basis of three hundred grains per group. Conditioning at temperatures of −1° to −5° C. and conditioning above 40° C. both resulted in a lower percentage of germination.

Grains conditioned when fully imbibed (44% water content) had progressed nearly two-thirds of the way toward germination (22 hours on moist surface) at the time of conditioning, since emergence began at about the thirty-fifth hour after the grains were placed in moist conditions; the groups with the 39% water content, however, were less than one-tenth of the way (3 hours on a moist surface). After conditioning at 10° C., grains germinated to essentially the same extent when conditioned at the two higher water contents (39% and 44%) when those incubated in light are compared with each other (78% and 80%); a similar comparison is made between those in darkness (84% and 85%). Germination of grains conditioned at 40° and 50° C. when fully imbibed tended to be greater in all cases than in the corresponding grains conditioned with 39% water. In both light and darkness, germination of the groups conditioned at 10°, 40°, and 50° C. exceeded that of the controls. This is an amplification of the response noted for grains conditioned with water content of 39% when incubated in darkness. After conditioning at 60° C. at both the higher water contents, a few grains germinated in light, none in darkness.

Thus many combinations of temperature conditioning and light or darkness promote higher germination percentages than those occurring in the control groups.

CONDITIONING-TEMPERATURE COEFFICIENTS.—It was considered worth while to obtain what may be termed conditioning-temperature coefficients for the grains conditioned at 50° and 60° C. and 60° and 75° C. Between 60° and 75° C. the Q_{10} was corrected for the 15° C. difference. The values presented are those for the suppression of germination by heat. Thus, in a comparison of the germination of grains conditioned dry at 60° C. with those conditioned at 75° C., the Q_{10} was about 2 for grains incubated in light; in darkness, a value was not obtainable on the basis of only three hundred grains because the germination percentage after conditioning at 75° C. was 0. When a thousand grains were conditioned at 75° C. and incubated in darkness, however, 0.5% germinated. Hence, the Q_{10} between the treatments at 60° and 75° C. for grains incubated in darkness was 20–25. Grains conditioned with a water

content of 27% had Q_{10} values of 1.3 in light and 1.9 in darkness between the conditioning temperatures of 50° and 60° C., but values of about 29 and 50 were obtained for the same light conditions between the 60° and 75° C. treatments. The latter were calculated on the basis of germination percentages of 0.3% in light and 0.1% in darkness after grains were conditioned at 75° C. For the two higher water contents no germination of grains conditioned at 75° C. was obtained even when tests were made on samples of one thousand to two thousand grains. In a calculation of the Q_{10} for grains conditioned with higher water contents at 50° and 60° C., the values were again found to be greater in dark-incubated groups than in those in light. The highest value, 375, was obtained for fully imbibed grains incubated in darkness, as based on 0.2% germination in the group conditioned at 60° C.

ENZYME ACTIVITY.—In investigations on the autolytic ability of heated and unheated grains, 1-gm. samples were ground coarsely, placed in 25 ml. of distilled water in test tubes, a layer of toluene added, and incubated at 37° C. for 8–10 days; the heated grains had been exposed to 60° or 75° C. for 1 hour while fully imbibed. The amount of solid matter remaining at the end of the incubation period was from 30 to 300% greater in the control groups than in groups previously heated.

The activity of the ámylolytic enzyme (probably an amylase) in these grains was also studied, using the time necessary for starch to be hydrolyzed to the achroodextrin stage (i.e., to give a colorless test with IKI) as a measure of activity (10). A filtered extract of ground, unheated grains (0.5 gm/10 ml water) carried an equal volume of a 2% solution of soluble starch to the achroodextrin stage in 20–30 hours at 30° C. In most cases the attainment of the end point was increased to 50, 100, or more hours in comparable preparations of grains conditioned at 60° C. or more for 1 hour either with 39% water or fully imbibed; this was also true of the two groups with lower water contents preheated at 75° C. Preparations of the wetter grains conditioned at 75° C. failed, in many cases, to reach the colorless end point after several hundred hours.

Oxidase activity was measured semiquantitatively by the use of 10% alcoholic solution of guaiacum applied either to ground samples of grains or to crushed, individual grains which were examined under low-power magnification of the compound microscope. The only distinction in results was in groups conditioned at 75° C. with the two higher water contents; these developed little or no blue coloration with the test solution.

These and other studies on enzyme activity give only indications of some of the possible changes in the grains associated, directly or indirectly, with the killing action of heat.

When grains with about 18% water content were conditioned at 75° C. for 1 hour, germination failed in either light or darkness. If, however, 3–4 ml. of $M/100$ glucose were supplied to populations of one hundred to three hundred grains incubated in light, some 3–9% germination was observed; in darkness, only 1–3% of the grains germinated. A number of feeding experiments of this nature were attempted with some of the intermediates in carbohydrate metabolism—pyruvate, citrate, succinate, etc.—but their polar nature probably prevented their entrance into the cells of the grains.

Sporobolus

The germination responses of grains of *S. airoides* to various temperature conditioning and light conditions are given in table 2 for two water contents. The first, 10%, corresponds to the air-dry condition; the second, 40%, to the fully imbibed condition. Upon imbibition the mucilaginous layer of the fruit coat of *S. airoides*, *S. corighti*, and *S. cryptandrus* swells, forming a gel in which much of the imbibed water is retained. Because of this the true water content of the grains themselves is not known.

Grains, when conditioned dry, showed responses very much like those of the corresponding crabgrass group (air-dry), except that the differences between those incubated in light and darkness were not so great. Although crabgrass showed little or no change in percentage of germination from that of the control (25° C.) when conditioned at 40° C., air-dry *Sporobolus* grains so conditioned germinated 5–10% more and imbibed grains 8–15% less than their controls. Stimulation of germination in grains heated dry was greater in light than in darkness; suppression of germination in imbibed grains was less in light. Again, it is apparent that the germination percentage can be improved by several combinations of light and temperature conditions.

On measuring the amylolytic activity of grain extracts as before, it was found that the hydrolysis of starch to the achroodextrin stage by preparations of untreated grain required 15–20 hours, while 50–100 hours were required by preparations of imbibed grains conditioned at 65° C.; hydrolysis did not occur in several hundred hours with preparations from grains exposed to 100° C. for 30–70 minutes.

Imbibed grains exposed to 60° C. for 1 hour failed to germinate in Petri dishes on a substrate of filter paper moistened with water. If, however, $M/100$ glucose or sucrose or $M/1000$ hydroquinone was added, 1–3% germination was observed after 125 hours incubation with a constant illumination of 300 foot-candles intensity. In darkness, no germination occurred. Other substances, including ascorbic acid, thiamin hydrochloride, riboflavin, etc., were without effect.

TABLE 2

PERCENTAGE GERMINATION OF SPOROBOLUS UNDER VARIOUS CONDITIONS

Water content of grain when conditioned	Incubated in	Conditioning temperature (° C.)					
		10	25*	40	50	65	100
10%	Light	57	61	71	75	78	0
	Darkness	65	62	67	71	71	0
40%	Light	57	58	50	33	0	0
	Darkness	70	62	47	28	0	0

* Controls.

Grains given the same heat treatment but planted in soil germinated 5–20%.

Dry grains exposed for 1 hour to 65° C. produced stunted seedlings when planted. After about 3 weeks, when the seedlings from control grains had emerged from soil to an average height of about 70 mm., seedlings from heated grains averaged about 45–50 mm. tall. After 12 weeks the control plants averaged 221 mm. in height, the plants from heated grains about 170 mm. Analysis of height distribution in each population showed that whereas, in the former groups, one-third were in the "tall" (280–380 mm.) class, only 5% of the plants from the latter population fell in this interval.

Flax

Under the conditions of the experiment neither the temperature treatment nor light exerted a perceptible influence on the germination of flax seed, except for the killing of imbibed seeds by exposure to 65° C. for 1 hour (table 3). Radish, under similar conditions, gave almost identical results.

TABLE 3

PERCENTAGE GERMINATION OF FLAX UNDER VARIOUS CONDITIONS

Water content of seed when conditioned	Incubated in	Conditioning temperature (° C.)				
		10	25*	40	50	65
8%	Light	97	96	100	99	88
	Darkness	98	94	99	95	95
42%	Light	90	95	95	89	0
	Darkness	89	91	91	88	0

* Controls.

Effects of brief exposures to 100° C.

Seeds of a number of species were employed in studies of effects of more or less extreme high temperature (100° C. or more); certain preliminary observations may be noted here. Of ten species used, including wheat, corn, radish, bean, pea, sunflower, flax, and several wild species, not one was capable of germinating when dry seeds were subjected to as little as 15–20 minutes at 135° C. or above. Further, when treated with guaiacum after such treatment, the cereals gave no oxidase or peroxidase test, although they are normally positive. For the three most resistant species the temperature which dry seeds could tolerate for 1 hour and still give at least 10% germination lies between 120° and 125° C. for wheat, and 110° and 122° C. for radish and flax.

In table 4 are given the responses of flax, radish, bean, and corn to several durations of 100° C. Corn and bean responded similarly; flax and radish constituted another set showing similar responses. Dry corn and bean seeds can tolerate no more than 20-minute exposure to this temperature and still attain 50% germination, while flax and radish

TABLE 4

PERCENTAGE GERMINATION OF FOUR SPECIES AFTER 100° C. TREATMENT

Duration of exposure 100° C. (Min.)	Incubated 100 hours in	Flax	Radish	Bean	Corn
0	Light	94	83	84	87
	Darkness	100	90	84	91
20	Light	60	76	63	69
	Darkness	75	87	69	86
40	Light	60	33	0	0
	Darkness	76	51	6	0
60	Light	6	9	0	0
	Darkness	57	48	2	2

germinated to a fair degree after 40- or 50-minute exposure. The two sets may also be distinguished on the basis of their responses to light; the less tolerant seeds germinated to almost the same extent in light and darkness, while the more resistant ones germinated decidedly better in darkness.

When treated in an identical manner, grains of wheat showed virtually no difference in germination between the control group and those exposed to 100° C. for 20–60 minutes.

When dry flax seeds were heated at 110° C. for about 65 minutes, germination in Petri dishes in the absence of light was no more than 30% in 230 hours (about 4% in 100 hours). On the addition of 3–4 ml. of $M/100$ ascorbic acid, some 48% germinated in 180 hours (20%

in 100 hours). When similarly treated groups were planted in clay loam soil, 35–45% of the seeds established seedlings in 2 weeks. Half of these seedlings showed some stunting, and several had double or triple epicotyls. When heated seeds were germinated in Petri dishes with extracts of the same soil (5–10 gm/50–100 ml water), as much as 55–65% germination was observed in 170–200 hours (germination of controls 95% in 100 hours).

Several abnormalities were noted in the roots of young flax seedlings. Some seeds germinated (if it may be so termed) by the protrusion of cotyledons through the chalazal end of the seed. Others produced hypocotyls with clubbed, brown root apices which failed to elongate. Lateral root development in this case was extensive. Another type of abnormality involved swelling of the seed to several times the normal volume when imbibed and the extension of the micropylar end of the seed for some time before emergence, giving the seed a flask-shaped appearance. Other abnormalities were noted. An analysis of the abnormalities in root growth induced by heat and ionizing radiations is planned by the writer.

Discussion

Any interpretations of the results herein reported must be of a tentative nature, owing to the extreme physical, chemical, and morphological heterogeneity of the seed. The treatments employed and the studies undertaken of the responses have not been reported previously as such, although this work may be said to embody several experimental approaches often used, such as those involved in heat-death investigation. Although no comprehensive analysis of the physiological bases for these observations can be given at this time, some suggestions as to the events occurring during the treatments can be made. Some of the data, however, lend themselves to no satisfactory explanation on the basis of present knowledge.

It is evident that, depending on the pattern of conditioning, germinating crabgrass grains manifest three different responses to light. Germination of prechilled grains, wet or dry, is inhibited by light; grains at room temperature or moderately heated respond indifferently to light; after conditioning at higher temperatures, germination tends to become light-obligate. The response of dry grains conditioned at 75° C., after which germination occurs only in the presence of light, is most striking.

If the response in the absence of light is considered a direct response to the conditioning treatments, then it can be said, on the basis of the evidence given here, that light can reduce the deleterious effects of the higher temperatures on germination. It has been suggested that light may, under certain conditions, increase enzyme activity *in vivo* (**8**). Amylase has received mention in this connection. The evidence accumulated in the last 50 years is not conclusive, but it is at least plausible to suggest that a higher percentage of germination in light than in darkness (i.e., an alleviation of heat injury by light) may be related to greater enzyme activity or zymogenesis in light. The writer cannot as yet substantiate this contention with quantitative data, but he has ascertained that light is capable of passing through the fruit coat of the crabgrass grain and, being incident on the storage and protoplasmic components of the grain, may initiate some photochemical reaction or reactions affecting metabolic processes. After an exposure of 75° C., even dry enzyme

preparations would show some decrease in activity. But seeds are not dry, and a water content of 9% in air-dry crabgrass grains could increase the rate of thermal inactivation of enzymes, thus permitting any stimulatory effect of light on the enzyme system of the seed to become apparent.

The large values obtained for temperature coefficients are probably indicative of the thermal denaturation of proteins (1). Certainly the grains with higher water contents heated to 60° or 75° C. have less amylolytic activity than untreated grains. It must be remembered, however, that no perfect correlation can be established between amylase inactivation and loss of viability. In addition, some of the groups failing to germinate still had high oxidase activity, and only the two groups receiving the most severe treatments had high oxidase activity, and only the two groups receiving the most severe treatments had little or none. Autolytic enzymes—pectases, cellulases, cytases, or others—had diminished activity. The ability of a few air-dry grains heated to 75° C. to germinate in the dark with glucose suggests that, in those grains which germinated, the only damage incurred through the treatment was the inactivation of the amylolytic enzyme, resulting in the cutting-off of the energy supply of the embryo by preventing starch digestion. The responses of grains which had been conditioned identically, except that they contained 18% water, further bears this out. The somewhat higher germination with both light and glucose suggests, perhaps, better utilization of glucose in light. Data on the effects of light on plant respiration are contradictory and inconclusive (7). CROCKER (3, 4) mentioned the light relations of *Oenothera* seeds and pointed out that these seeds, whose germination is favored by light, lose their light-sensitivity when the seed coat is pricked. The present author has also found this to be true for *O. biennis*. This had led to the suggestion that light-sensitivity may be linked to gas exchange. The writer has also observed that exposure of freshly collected seeds of evening primrose to 50° C. for 40–90 minutes improved their germination in darkness.

It was pointed out that many conditioning treatments resulted in germination percentages higher than those which were observed in the controls. It has long been known that sudden changes of both low and high temperatures can stimulate biological processes (2, 6). HEILBRUNN and others have proposed that the stimulatory effects of temperature changes may be a result of changes in protoplasmic viscosity. Much of the work on cleavage rate of the sea-urchin egg (6) has supported this contention. The methods employed in measuring the viscosity of either plant or animal protoplasm are impossible to apply to plant embryos; thus it can only be conjectured that this phenomenon occurred during the treatments here reported. The storage of some fruits and vegetables at low temperatures for brief periods has been reported to promote the accumulation of soluble carbohydrates and to stimulate respiration (7). Considering the methods of the present experiments, it is not likely that the promotion of germination by conditioning grains at 10° C. had such a basis, but experimental evidence has yet to be collected. Stimulation of germination after conditioning at high temperatures results, in all likelihood, from increases in chemical activity, accelerating the mobilization of reserve materials and the release of energy. Again, the stimulatory effects of light, or light plus

various conditioning temperatures, may be a result of increases in enzyme activity, respiratory activity, permeability of cells to oxygen (7), a combination of these, or from causes yet unknown.

Much of the preceeding discussion, centered around the responses of crabgrass, applies equally well to the responses of *Sporobolus*. Specific differences in the nature of tissue proteins, insulation of the embryo by maternal tissues, as well as such factors as age, previous history, water content, etc., would account for quantitative differences in the responses of the two grasses. The efficacy of hydroquinone, as well as carbohydrates and planting, in promoting limited germination of wet grains after exposure to 60° C., suggests that in the few grains that did germinate some reversible oxidation-reduction reaction has occurred.

In addition to the germination responses the stunting of the seedlings and plants produced from heat-treated grains is noteworthy. The manner in which the heat treatment operated to limit growth is not known. Contributing factors may include thermal inactivation of enzymes, denaturation of tissue proteins, or may be of a more specific nature, acting on particular growth substances. In some actively growing plant tissue (corn seedlings), brief heating to 48° C. for 30 minutes increases the rate of auxin inactivation and, correspondingly, decreases the growth rate (13). At temperatures such as those employed in the present work, auxins, their precursors, or other growth factors in seeds could be inactivated. The writer has observed considerable (15–40%) stunting in young corn plants grown from grains which had been previously exposed to 100° C. for 30–40 minutes while dry. Whether or not this stunting is a result of destruction of auxin cannot be said at present, but it is not an unreasonable suggestion that the inactivation of growth factors by heat, even in air-dry seeds, accounts for the stunted condition of young seedlings.

The relative insensitivity of flax (and radish) to the conditions employed was notable. Evidence on hand, however, indicates that with prolonged chilling (100 hours or more) of dry flax seeds, the germination percentage increased. Further, whereas the flax seed cannot be said to be sensitive to light on the basis of the present work, the picture is altered considerably when light is employed on wet seeds for brief periods (10 minutes to 2 hours). Germination and early seedling growth are thereby improved. Prolonged chilling was observed to diminish the differential responses of seeds to light and darkness when the illumination was brief. Radish also shows differential germination in light and darkness under brief illumination. The "induction" of a light-sensitive condition in these species will be considered as it is related to conditioning at 100° C.

The separation of the four species, corn, bean, flax, and radish, on the basis of oil content (7) agrees with their separation on the basis of responses to the extreme heat treatment. Whether this is coincidental cannot be established at present. The high resistance of wheat would weaken any conclusion that high oil content increases heat resistance on the basis of so few species. A relation of oil content to the effects of temperature on the organism has, however, been postulated by several investigators (2, 6). The effect of ascorbic acid and soil extracts on flax at even higher temperatures may well be a function of the activity of this vitamin and some com-

ponents of soil in oxidation-reduction reactions. If some of the injurious effects of heat are a result of oxidation, the relative insensitivity and higher resistance of oily seeds to high temperatures might be a result of antioxidant activity of the unsaturated fatty acids in flax and radish (7). The far greater tolerance of wheat to the heat treatment is of great interest but cannot be accounted for at present.

No explanation can be provided for the increased inhibition by light of germination which was observed in seeds heated at 100° C. Again, the problem of the action of light arises. Conceivably, with such severe treatment substances capable of interacting with light to inhibit germination could be produced. WEINTRAUB (12), in a consideration of lettuce seed germination, pointed out that stimulation of germination by light is manifested only under suboptimal conditions. The responses of some seeds—crabgrass, *Sporobolus*—to light after high-temperature conditioning conform well to this concept; light promotes germination after conditioning of the grains at elevated temperatures which are certainly injurious and consequently produce a suboptimal condition within the grain. It is of interest, however, that flax and radish seeds on exposure to a conditioning temperature of 100° C. for 20–60 minutes manifest the reverse response; indeed, on prolonged exposure to this temperature, there is virtually no germination in the presence of light. There is insufficient evidence on the basis of the small number of species here reported upon to permit a conclusion as to which of these two types of responses to high-temperature conditioning (photostimulation or photoinhibition of germination) is more characteristic of seeds in general. It must be remembered that WEINTRAUB wrote of the relation of light to exogenous chemical germination inhibitors, while the present paper deals with physical conditioning of the seed.

The response of flax to a conditioning temperature of 110° C. demonstrates, among other things, the great difference which may exist between percentages of germination in Petri dishes and in soil. The soil was able to supply substances, oxidizing or reducing agents, capable of increasing the rate of germination and final percentage—i.e., capable of facilitating a partial recovery of the seeds from the effects of heat. Even in soil some seeds were incapable of emerging. The still more improved germination which was observed in Petri-dish culture with added soil extract bears this out. In the last instance, which provided the seeds with the beneficial agents in soil but removed its physical resistance, a far higher percentage of germination was possible. The difference between percentage of emergence from soil and percentage of germination in soil extract probably is accounted for by those seeds lacking in the materials for energy and cell substance needed to overcome the mechanical resistance of the soil.

Abnormalities in young flax seedlings may be, in part, a result of damage to the root apex, which is extremely sensitive (5).

Summary

1. The effect on germination of brief conditioning at various temperatures of the grains or seeds of several species of plants was studied, and the effects of such conditioning on light-sensitivity, enzyme activity, and, in two instances, on subsequent growth were observed. Grains of crabgrass and *Sporobolus airoides* and seeds of flax and radish were conditioned at temperatures ranging

from 10° to 65° or 75° C. for 1 hour while possessing various moisture contents from air-dry (9–10%) to fully imbibed (40–44%). Air-dry grains of corn and wheat, seeds of radish and flax, and seeds of other species were conditioned at temperatures of 100° C. for 20, 40, or 60 minutes, or exposed to higher temperatures.

2. Crabgrass and, to a lesser extent, *Sporobolus* exhibited what is termed a threefold response to light. After conditioning at 10° C., germination percentage was higher in darkness than in light; after conditioning at higher temperatures (50° C. or more), germination became relatively greater in light—in one case, light-obligate. Responses to light varied with differences in water content as well as with temperature of pretreatment.

3. After treatments which resulted in the suppression of germination, the activity of enzymes and the degree of autolysis were diminished. Loss of viability through heat treatments is not in all cases correlated with the loss of oxidase activity.

4. Treatments preventing germination of *Sporobolus* could be overcome in some proportion of the grains by the addition of glucose, sucrose, or hydroquinone; light, glucose, or light and glucose were similarly effective on crabgrass grains.

5. Grains of *Sporobolus* conditioned at 60° C. for 1 hour and then planted in soil produced stunted plants; stunting was observed at the third and twelfth weeks after planting.

6. Many instances were observed in which temperature conditioning acting alone or with light promoted more germination than in the control groups. This is discussed in terms of increased chemical activity, the possible effects of light on enzyme activity, and the generally observed stimulatory effect of sudden changes in temperature. The possible relation of the latter point to changes in protoplasmic viscosity is mentioned.

7. In all cases a combination of high water content and high conditioning temperature was deleterious to germination. The upper limit of heat tolerance for seeds of a number of species heated dry is less than 135° C. for 15 minutes. The most resistant species studied were wheat, flax, and radish; of these, wheat is most tolerant to high temperatures and is little affected by as much as 60 minutes' exposure to 100° C.

8. The relative insensitivity of flax and radish seed over a wide range of conditions was found to be of interest. This may be related to the high oil content of these seeds. They are more resistant to treatment at 100° C. than bean and corn, which are both low in oil. The responses of flax and radish to increasing time of exposure to this temperature are similar. Light is clearly inhibitory to these two species after the heat treatment, while it makes little or no difference in the germination of corn and bean.

9. Flax seeds heated 110° C. for 65 minutes exhibit the following characteristics: (*a*) poor germination in Petri dishes with water, (*b*) much improved germination in soil or soil extract, (*c*) improved germination with ascorbic acid, (*d*) certain abnormalities in the roots of young seedlings, and (*e*) a stunting of plants grown in soil from seeds so treated. It is suggested that the efficacy of certain agents in overcoming partially the injurious effects of heat is related to their oxidation-reduction activity.

10. The heat resistance of flax and radish is discussed briefly in terms of a protective action of the oils present, pos-

sibly an antioxidant function of the unsaturated fatty acids.

11. The data here presented are of limited value in a strictly quantitative sense but demonstrate that brief heat and cold treatments can have numerous effects on the physiological system of the seed. Some of the factors limiting the reproducibility of results are mentioned, and the difficulty of interpretation of the results, owing to the complex and heterogeneous nature of the seed, is pointed out.

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EFFECT OF GROWTH-REGULATING SUBSTANCES ON THE WATER-RETAINING CAPACITY OF BEAN PLANTS

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Introduction

It has been reported earlier that the water content of some plant tissue and parts of plants increased following treatment with either indoleacetic acid or 2,4-dichlorophenoxyacetic acid (**3**, **4**, **5**, **8**). The present experiments were initiated during 1947 to study the effect of various plant growth-regulating chemicals upon the water content and water-retaining capacity of entire plants and of detached plant parts.

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Experimentation

In preliminary trials with Black Valentine bean seedlings the water content of the tops (above-ground portion) was significantly increased and the rate at which water evaporated from the freshly detached tops was reduced after treatment with growth-regulating substances. These effects on beans grown in a green-

house were tested by dipping their entire tops into aqueous mixtures that contained separately 100 p.p.m. of the different growth-regulators. Sufficient ethyl alcohol to make a final concentration of 0.5% was used to disperse the growth-regulators in the water. Approximately 21 hours after treatment the stems were severed near the ground level. Each detached top was weighed and immediately hung up to dry in static air at room temperature. The samples were weighed at subsequent intervals to determine the rate of water loss. Finally they were oven-dried and their total moisture content determined. This experiment was repeated three times, and in each instance treated plants exhibited a reduced rate of water loss in comparison with untreated plants (table 1). 4-Chlorophenoxyacetic acid (4-Cl) and 2,4,5-trichlorophenoxyacetic acid were most effective in this respect, while 2,4-dichlorophenoxyacetic acid was less effective. Greatest reduction in rate of water loss occurred during the early part of the drying period.

TABLE 1

WATER LOSS OF DETACHED TOPS OF BEAN SEEDLINGS UNTREATED OR TREATED WITH GROWTH-REGULATING CHEMICALS. FIGURES ARE AVERAGES OF FIVE INDIVIDUAL MEASUREMENTS AND SHOW PERCENTAGE LOSS, BASED ON INITIAL WATER CONTENT, DURING SPECIFIED PERIODS BEGINNING AT HARVEST, WHICH WAS 21 HOURS AFTER TREATMENT

Hours after harvest	Untreated control	Treated control (0.5% alcohol)	NAA*	2,4-D	NOAA	4-Cl	2,4,5-T	IAA
	Experiment I							
4	20.9	19.9	16.1	16.8	17.2	14.2	14.4	15.8
70	67.9	66.1	66.1	70.9	65.8	63.9	67.1	64.1
	Experiment II							
4	22.9	21.5	16.7	16.1	16.9	14.5	15.0	17.0
24	49.8	47.7	43.2	42.5	44.5	39.5	44.3	44.2
48	65.6	63.8	59.9	60.7	61.6	56.9	63.7	61.7
	Experiment III							
4	22.4	22.0	15.1	15.7	14.9	14.7	15.6	17.9
24	47.8	47.2	38.4	41.4	39.2	40.1	42.2	41.4
48	62.1	60.8	55.2	60.5	55.4	59.0	62.0	55.1

* NAA, alpha-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 4-Cl, 4-chlorophenoxyacetic acid; NOAA, beta-naphthoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; IAA, indoleacetic acid.

At harvest the percentage of moisture in the tops of plants treated with 4-Cl was 1.4, 1.6, and 1.3% greater, respectively, than for untreated plants in the three experiments. In these same experiments the moisture content of plants treated with 2,4,5-trichlorophenoxyacetic acid was increased 2.0, 2.1, and 2.0%.

Smaller increases were found in plants treated with the other growth-regulators.

Application of 4-Cl to the stems and leaves before the plants were harvested also retarded water loss from the entire harvested plants. Young, rapidly growing bean seedlings were treated with 4-Cl as described above. One day later their roots were washed free of soil and the subsequent rate of water loss from the entire plant determined. Sixty per cent more water evaporated from the untreated plants than from the treated ones during the first $1\frac{1}{2}$ hours after harvest. This difference in rate of water loss decreased, however, and at the end of 24 hours the untreated plants had lost approximately 7% more water than had treated ones.

Moisture loss from detached bean fruits was retarded by applying 4-Cl to them a few days prior to harvest. Black Valentine bean plants grown in a greenhouse during December, 1947, and January, 1948, were used. After several fruits on each plant had reached a marketable size, all smaller ones were removed. Fruits on half of the plants were then momentarily immersed in an aqueous mixture containing 1000 p.p.m. of 4-chlorophenoxyacetic acid and 1% of Tween 20. For comparison, fruits on the remaining plants were left untreated. At intervals of 1, 2, and 3 weeks after treatment, samples of both treated and untreated fruits were harvested and weighed. Those collected in the first and second harvest were immediately dried and their moisture content determined. Those collected during the third harvest were spread in a single layer on a greenhouse bench and allowed to dry.

The average fresh weights of treated fruits harvested 1, 2, and 3 weeks after treatment were, respectively, 11.2, 10.1, and 11.4% greater, and they contained 2.3, 8.7, and 14.0% more moisture than did fruits from untreated plants harvested at comparable intervals. Of fruits treated and left attached for 3 weeks, approximately 62% were green and turgid when picked, while only 8% of untreated ones were green, the rest being dry and brown. Of the fruits laid out to dry, approximately 33% of the treated ones were still entirely green, or partly so, at the end of 1 week, whereas none of the untreated ones was completely green and only 5% were partially green. Although treatment resulted in an increase in the average fresh weight per fruit, the number of seeds per fruit was reduced. On a dry-weight basis, seeds that developed in treated fruits weighed an average of 22% less than did seeds from untreated ones; while the dried hulls (without seeds) of treated fruits averaged approximately 14% heavier than did hulls from untreated fruits. With respect to water loss, the rate of evaporation from treated fruits after harvest was definitely retarded (table 2).

In other tests, sprays of aqueous mixtures containing 0, 50, 250, 500, or 1000 p.p.m. of 4-Cl were applied to attached bean fruits. The acid was dispersed in water with the aid of sufficient Tween 20 to make 1% of the co-solvent at each concentration of growth-regulator. At the time of treatment approximately three fruits on each plant had reached marketable size. There were also many of smaller size, some 1 inch or less in length. All were sprayed and allowed to remain attached for a period of 4 days, after which those of marketable size were harvested. Each treatment was replicated five times, with ten plants per replication. Fruits from each replication were spread out in a single layer on paper in a greenhouse and the rate of water loss

during the following 17 days determined.

Application of 50 p.p.m. of the acid did not affect yield on the basis of dry weight of fruits. Concentrations of 250, 500, and 1000 p.p.m., on the other hand, reduced yields a significant amount and in proportion to the concentration applied. This apparently resulted from a retarding effect of the higher concentrations of acid on the growth of the partially developed fruits. The greatest reduction in yield (in the case of the 1000-p.p.m. concentration) amounted to 44% of the yield of the control plants.

There was a marked effect of treatment on the number of detached fruits that remained green and turgid during the 17-day drying period. All the control fruits shriveled, turned brown, and became completely dry and brittle during this period. In contrast, an average of 25, 27, 40, and 65% of those treated, respectively, with 50, 250, 500, and 1000 p.p.m. of the acid remained green in color and pliable.

The acid sprays reduced the rate at which water evaporated from the fruits after harvest. The effect was highly significant on a statistical basis and remained so during the entire 17-day period. Maximum retardation of water loss occurred, however, during the first 6 days after the fruits were picked and laid out to dry (table 3).

Several experiments similar to that described above have subsequently been carried out. In all these, 4-Cl has consistently brought about a retardation of the loss of moisture from harvested bean fruits. An example of the effect obtained is shown in figure 1.

Wax coatings are sometimes used commercially to reduce water loss from

TABLE 2

EFFECT OF 4-CHLOROPHENOXYACETIC ACID (DIP TREATMENT OF ATTACHED PODS) ON WATER LOSS OF BEAN FRUITS AFTER HARVEST. FIGURES REPRESENT AVERAGES OF SIX REPLICATIONS (APPROXIMATELY THIRTY FRUITS EACH) OF EACH TREATMENT.

Days after harvest	Percentage water loss based on content at harvest	
	Control	Treated
0.3	22	18
0.7	34	25
1.3	41	34
2.0	56	49
3.0	93	81
4.0	95	87
5.0	95	92
6.0	97	95
10.0	99	99

TABLE 3

EFFECT OF 4-CHLOROPHENOXYACETIC ACID SPRAYS ON WATER LOSS OF BEAN FRUITS. FIGURES REPRESENT PERCENTAGE LOSS ON BASIS OF WATER CONTENT AT HARVEST

Treatment (p.p.m.)	Days after harvest						
	1	2	4	6	8	10	17
Control	8.5	32.3	49.9	59.9	84.3	95.3	98.5
50*	5.8	24.8	42.2	55.0	79.7	91.2	97.5
250	5.2	21.5	37.9	51.6	79.1	90.9	97.7
500	5.3	21.6	34.8	48.6	76.0	87.4	95.9
1000	5.9	21.7	33.2	43.7	73.1	84.6	93.4

* All treatments significantly reduced water loss during 17 days by ratio of 1:19 or better.

fruits and vegetables after they are harvested. 4-Chlorophenoxyacetic acid was added to wax-emulsion sprays to determine whether the addition of this chemical would produce a mixture that was more effective than the wax emulsion alone in retarding water loss from bean fruits. A wax emulsion (Brytene) to which were added 1% of Tween 20 and 400 p.p.m. of 4-chlorophenoxyacetic acid was sprayed on attached Black Valentine bean fruits of marketable size grown under greenhouse conditions. For comparison, an equal number of fruits were sprayed with the wax emulsion containing 1% of Tween 20 without the acid added, and others were left untreated. Each treatment was replicated three times. Five days later the fruits were harvested and stored at room temperature.

FIG. 1.—Black Valentine bean fruits. *Right*, sprayed with aqueous mixture containing 400 p.p.m. 4-chlorophenoxyacetic acid and 0.5% Tween 20 4 days before harvest; *left*, comparable unsprayed fruits. Photographed after holding 10 days on greenhouse bench.

During the first week of storage unsprayed fruits lost approximately 25% more water than did those sprayed with the mixture of wax and growth-regulator. Addition of the growth-regulator to the wax emulsion increased the effectiveness of the mixture in checking water loss. During 2 weeks of storage unsprayed fruits lost 77.2%, those sprayed with the wax emulsion alone 67.9%, while those sprayed with the wax emulsion plus the acid lost only 48.0% of their original fresh weight. Fruits sprayed with the wax and growth-regulator mixture retained their original color longer and appeared to be more turgid after storage than did untreated ones or those sprayed with the wax emulsion alone.

Discussion

From a physiological standpoint the observed increase in water-absorbing capacity of plant tissues may have resulted in part from an increased osmotic concentration of the cell sap of tissues that are sensitive to the growth-regulators. It is known that such mineral elements as iron, copper, magnesium, manganese, phosphorus, potassium, aluminum, and boron accumulate in tissues of bean stems under the effect of indoleacetic

acid (4). In general, the effect of such growth-regulators as indoleacetic acid and the phenoxy compounds has been to accelerate the rate of hydrolysis of complex carbohydrate constituents and bring about the accumulation of sugars (**1, 2, 6, 7, 10**). Degradation of some of the complex nitrogenous compounds in bean plants apparently also results from the application of these chemicals (**6, 9**). Thus, accumulation of minerals and unelaborated carbohydrate and nitrogenous materials would tend to increase the effective osmotic concentration of the cell sap and, in part at least, account for the increased water absorption observed in these experiments. It is suggested that the increased water-retaining capacity of treated tissues may have resulted from a change in the water relations of protein constituents in the cells.

It is stressed that results presented here should not be interpreted as a recommendation for practical use of growth-regulators in retarding water loss from food products. More information is needed regarding toxicity of these chemicals to human beings and regarding their effect on the growth and yield of plants under field conditions. Studies are in progress to determine whether 4-Cl is toxic to animals when encountered in the amounts used here.

Summary

1. 4-Chlorophenoxyacetic acid (4-Cl) was applied as a spray or a dip treatment to attached snap-bean fruits 4 days before the usual harvest date. Effect on their subsequent development and on certain after-harvest changes in the fruits were studied.

2. One thousand parts per million of 4-Cl retarded maturation of attached fruits. Fresh weight of treated fruits was 11.2, 10.1, and 11.4% greater than that of untreated ones when harvested 1, 2, and 3 weeks after treatment, and the treated fruits contained 2.3, 8.7, and 14.0% more moisture, respectively, than did the untreated ones. Application of the acid brought about a decrease of 22% in final dry weight of seeds produced, but the dry weight of pods of treated fruits was 14% heavier than that of untreated fruits at maturity.

3. Spray applications of 4-Cl at concentrations ranging from 250 p.p.m. to 1000 p.p.m. to attached fruit reduced the final yield of fresh fruit—with the most concentrated spray, by 44%. A 50-p.p.m. spray did not significantly reduce yield under the conditions used.

4. Spray applications of 4-Cl at concentrations ranging from 50 p.p.m. to 1000 p.p.m. to attached fruits increased their water-retaining capacity when they were harvested and stored. The acid retarded the rate at which color changes occurred in harvested and stored fruits.

5. Application of 4-Cl (400-p.p.m. spray) in a wax emulsion to the attached fruits was more effective in retarding evaporation of moisture from the fruits following harvest than was an equal concentration of the acid in aqueous spray. During the first week of storage, fruits sprayed with the wax–growth-regulator mixture lost approximately 25% less moisture than did unsprayed ones. Further investigations are necessary before application of 4-Cl can be considered as a practical means of improving the storage quality of snap beans.

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HERBICIDE EFFECTS IN CONNECTICUT VEGETATION, 1949

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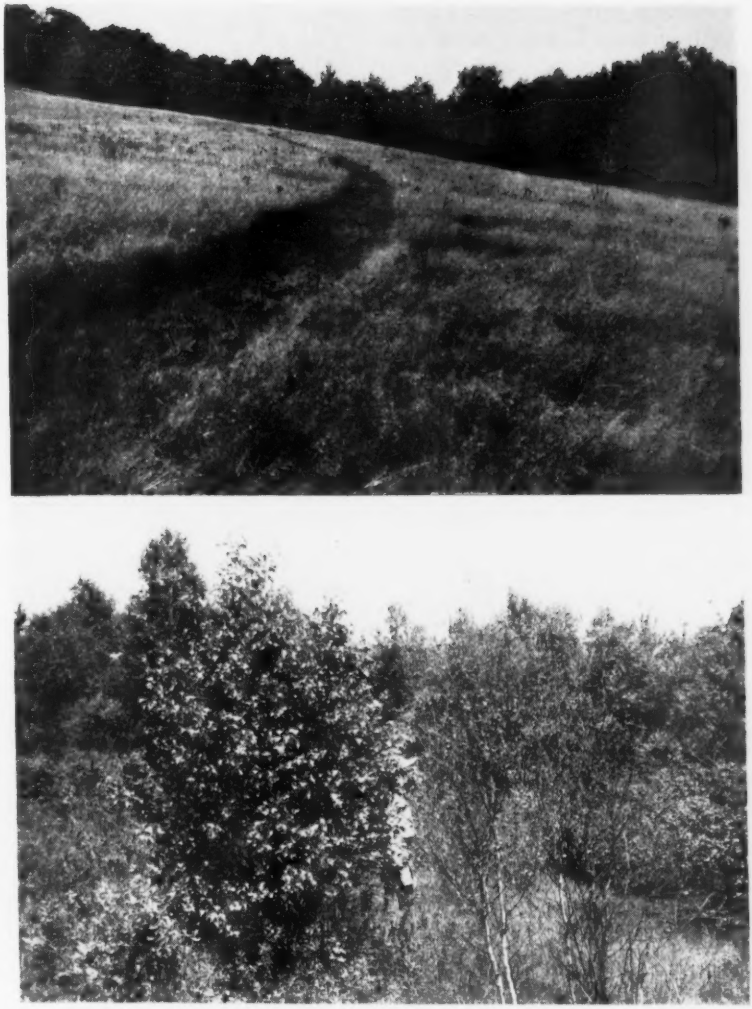
Introduction

This paper is the fourth and penultimate report on herbicidal studies being carried out at Aton Forest, northwestern Connecticut. The first three (**1**, **2**, **3**) reported effects for 1946, 1947, and 1948, respectively, resulting from various spot-treatments (mostly sprays on foliage) of 116 species, using various concentrations of several formulations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in water. This fourth report presents the data for 1949, from foliage spraying with 250 gallons of aqueous solutions, and from wood and bark treatments with various concentrated solutions, involving the equivalent of approximately 2 acres of continuous dense brush. The results in 1949 pointed so significantly to the advantages of bark treatment over foliage spraying for the control of woody plants that all foliage spraying of such plants was halted in midsummer as being economically impractical.

Three miles of forest trails, 5 acres of pine forest, 20 acres of seminatural grasslands (fig. 1), and 15 acres of seminatural shrublands are now under herbicidal control. These studies on herbicides are part of a larger long-term project involving the degrees of phytosociologic equilibrium that come into existence under conditions in which certain elements of the flora are eliminated. Three types—relatively stable grasslands, shrublands, and pine forests—have at present been produced by the herbicidal elimination of: (*a*) all woody plants and rank herbs, (*b*) all trees, and (*c*) all trees except white pine, respectively. Reports on the composition and structure of these plant communities will appear at later dates.

Experimentation and discussion

All herbicides used are commercially available and included: American Chemical Paint Company's mixture of butoxy ethanol esters of 2,4-D and 2,4,5-T (Weedone Brush Killer 32); Dow Chemical's isopropyl ester of 2,4-D (Esteron 44), isopropyl ester of 2,4,5-T (Esteron 245), and an equal mixture of both (Es-



FIGS. 1, 2.—Fig. 1 (*above*), herbicide-induced grassland. Last plowed and planted pre-1926; mowed annually through 1944, at which time brush was abundant. Dark plants in grassland are remaining unsprayed *Solidago rugosa*. Strip in left foreground is part of mile-long mowed transect. Photographed on October 1, 1949. Fig. 2 (*below*), gray birches on right killed by spraying of basal bark with 20% D-T on December 2, 1948. Trees leafed out but browned and dried before leaves were fully expanded. Untreated birches to left. Photographed on July 26, 1949.

teron Brush Killer); Sherwin-Williams' butyl and alkyl esters of 2,4-D (Weed-No-More), of 2,4,5-T, and a mixture of both (Sherwilkil no. 2); and Socony-Vacuum's Sovasol no. 5. All these preparations (except the Sovasol) are in oil and are miscible with water for actual application.

Herbicidal techniques used in the 1949 season, each of which will be discussed separately, were as follows: spraying of foliage, winter spraying of total plants, applications to herbaceous stems, applications to stumps, applications to trunk wounds, and applications to bark of trunk bases.

Spraying of foliage

This treatment was directed mainly toward the remaining resistant and overlooked, unwanted plants in 20 acres of seminatural grassland, plus various small areas of brush and rank herbs that were treated for the first time. The major problem was a comparison of the relative effects of equal concentrations of 2,4,5-T and of 2,4-D–2,4,5-T mixtures. Half the acreage was spot-treated with a 0.5% (5000 p.p.m.) emulsion (popularly, and herafter, called a "solution") of 2,4,5-T esters in water; the other half, with the same concentration of a mixture of equal parts of 2,4-D and 2,4,5-T esters in water (hereafter referred to as D-T). Solutions of butoxy ethanol, propyl, alkyl, butyl, and a mixture of butyl and propyl esters were used. Spraying began on April 30 (when buds of trees were showing green but not yet broken open) and continued intermittently until August 17 (before any autumn coloration, although the drought of the summer had already resulted in yellowing of some tree foliage). All spraying was done with 2-gallon knapsack droplet sprayers. Mist-spraying was not used because of the inability to control drift of mist onto adjacent plants.

Results of these tests must be evaluated in the light of three previous seasons of spraying on most of this acreage, with up to five sprayings per year on some plants. Cumulative effects on some individuals, for example, may be beginning to become obvious. There were no observable differences at the inception of the winter of 1949–1950 between plants treated with 2,4,5-T and those treated with the lower-cost D-T mixture. No differences were noticeable among the effects of the different kinds of esters.

Although workers are still reporting lesser kill in shade than in light, and lesser kill in early spring than when midsummer foliage is fully expanded, studies here did not indicate this relationship. With the solutions and concentrations used, season-end kill was equal or slightly greater in shade and apparently equal for various degrees of foliage maturity. The rather frequent occurrence of a rapid browning and crisping of the foliage (rather than epinasty and a gradual discoloration of foliage), followed by regrowth later in the season, leads one to infer that the solutions this year may have been strong enough to burn the foliage and trap the chemicals within it rather than to allow their effects to spread gradually through the plant. Consequently, it may also be inferred that a weaker solution may be equally or more effective. Actually, no herbicidal end-effects were noticeable in 1949 with the 0.5% solution that were not similar to those obtained in 1948 with a 0.25% solution (taking into consideration the apparent cumulative effect from previous years on some individuals).

Relative resistance of 116 species to these formulations as tested in 1948 was essentially the same in 1949, and a complete listing is not here repeated. Pros-

trate species of blackberries (*Rubus* spp.) are apparently killed by a single spraying. One group of resistant species is now under control, possibly as much from the accumulated effects of sprayings of previous years as from the spraying of this season. This group includes red maple (*Acer rubrum*),[1] spreading dogbane (*Apocynum androsaemifolium*), New York fern (*Dryopteris noveboracensis*), lady fern (*Athyrium filix-femina*), hay-scented fern (*Dennstaedtia punctilobula*), winterberry (*Ilex verticillata*), bellwort (*Uvularia sessilifolia*), cinnamon fern (*Osmunda cinnamomea*), interrupted fern (*Osmunda claytoniana*), some individuals of white pine (*Pinus strobus*), some individuals of choke-cherry (*Prunus virginiana*), nightshade (*Solanum dulcamara*), and loosestrife (*Lysimachia ciliata*). It cannot be said, however, that such killing is economically feasible from the standpoint of material and labor costs. Several species of this group spread by underground rootstocks, so that their elimination to the last individual must be considered in the costs of producing a stable grassland if such is wished.

A second group of species includes those for which 4 years of foliage spraying leave them in a weakened condition from which they would still recover in 1 or 2 years. This group includes milkweed (*Asclepias syriaca*), beech (*Fagus grandifolia*), white ash (*Fraxinus americana*), low juniper (*Juniperus communis* var. *depressa*), red cedar (*Juniperus virginiana*), mountain laurel (*Kalmia latifolia*), sensitive fern (*Onoclea sensibilis*), trembling aspen (*Populus tremuloides*), bracken (*Pteridium aquilinum*), apple (*Pyrus malus*), live-forever (*Sedum purpureum*), hemlock (*Tsuga canadensis*), and arrowwood (*Viburnum dentatum*). Several of this group also are species which spread by underground rootstocks, and, for these cases also, the cost of their total elimination must be considered in the costs of producing a stable grassland. The woody plants of both these groups can probably be controlled by basal applications to the bark of the trunk (*q.v.* below); *Asclepias* can probably be eliminated by persistent painting of stems (*q.v.* below); and *Sedum*, by a stronger spray on the foliage. The remaining species are all ferns which today form the major herbicidal problem for any long-term conversion to grassland. Three species of fern common everywhere in the northeastern United States—*Dennstaedtia*, *Onoclea*, and *Pteridium*—appear to have potentialities for spreading and completely replacing the grassland. Consequently, a more effective chemical formulation for control of ferns is needed before conversion to grassland can be considered commercially practicable.

By the midsummer of 1949 it was observed that some root systems of aspen, ash, apple, choke-cherry, and even the relatively sensitive hardhack (*Spiraea latifolia*) were continuing to send up new shoots despite 4 years of spraying. This situation (coupled with the highly successful results of applications to the basal bark of trunks) led to the cessation of all further spraying of the foliage of woody plants. Barring new and contrary evidence, no foliage spraying of woody plants will be resumed in 1950 with the chemicals now available.[2]

Winter spraying of total plants

Several tests were made in the cold dormant season on woody plants (deciduous and evergreen) less than 1.5 m. in

[1] The Latin nomenclature has been changed by the editor to that of Gray's Manual, 8th ed., against the wishes of the author. The common names are those which in the opinion of the author are actually in common use.

[2] No foliage spraying has yet been resumed. August 5, 1950.

height, using low-volume mixtures of high concentration.

Indicative data were obtained on three otherwise resistant species, white pine, red maple, and low juniper, using a 5% solution of isopropyl 2,4,5-T on April 10. Of approximately thirty white pines (of which several were sprayed only on one side, several others only on the lower half) all were yellowish by mid-May and all were dead by late summer. Three hundred red maples along a forest trail were sprayed. These trees averaged 0.5 m. in height and formed a solid cover over the ground. They had been treated with a foliage spray in 1948: this resulted in stem curvatures, followed by regrowth and apparently complete recovery. Following the spraying in 1949 with 5% concentration, these maples leafed out in May, but all of them appeared dead a month later, and apparently none has survived. Low juniper has heretofore been completely resistant to foliage sprays, with effects at the most a temporary yellowing. Of two individuals with a spread of 0.5 m. in diameter, both began to yellow in late May and were dead by midsummer.

Mountain laurel has shown itself completely resistant to all previous foliage sprays during the summer; these have caused only temporary stunting and curvature of foliage. On April 17, 1949, a 5% D-T solution was applied to about twenty-five plants, parts of plants, and stumps and sprouts of plants previously cut down. By early fall all sprayed parts appeared completely dead.

Lycopodium complanatum var. *flabelliforme*, previously resistant to foliage sprays, was killed successfully by 5% sprays of esters during the cold dormant season.

Although the complete success of this one-spray treatment on hitherto troublesome species is worthy of record, other tests (*q.v.* below) would indicate that spraying of the entire plant may not be necessary, especially if a quick kill is not desired. Additional tests now indicate that treatment of (*a*) the lower trunk, lower branches, and foliage, or of (*b*) the lower trunk alone, may be sufficient for complete killing.

Applications to herbaceous stems

The only herbaceous species under investigation so resistant as apparently to require treatments of the stem rather than of the foliage is *Asclepias syriaca* (milkweed). In 1948 approximately two hundred stems forming a colony were painted in early August with 35% solutions of D-T and of 2,4,5-T (**3**:251). All stems yellowed slowly and were dead 6 weeks later, with no resprouting. In the spring of 1949 about seventy-five shoots appeared in the colony, all small, and probably from midpoints on the underground shoots between last year's treated stems. These were painted similarly, as were a few shoots appearing sporadically through the season. Killing of the colony, with all its underground stems, is not yet assured. Although this procedure for eliminating milkweed is costly of time and material, no other means is yet known for controlling this aggressive and densely colonizing herb.

Applications to stumps

Approximately 1000 small stumps up to 7 cm. in diameter were treated with solutions of various 2,4-D and 2,4,5-T esters, in concentrations from 25 to 35%, and through winter and summer months. The entire stump was painted or sprayed immediately, or within 2 weeks, after cutting. Species included gray birch (*Betula populifolia*), paper birch (*Betula papyrifera*), speckled alder (*Alnus rugosa*), willows (*Salix rigida, discolor,* and *bebbi-*

ana), tall blueberry (*Vaccinium corymbosum*), aspen, red maple, shadbush (*Amelanchier canadensis* and *arborea*), and fetterbush (*Lyonia ligustrina*), all of which were in the lines of new trails being cut through brushlands. Killing appeared effected in all instances. In addition, a special test was made on red maple in which 252 shoots (1–3 m. tall), involving 182 clumps, were cut with a brush-hook on December 8, 1948, and the stumps painted the same day with a 35% mixture of equal parts of butyl 2,4-D and isopropyl 2,4,5-T. None of these 182 plants sprouted the following spring or through the 1949 season; at the end of the 1949 season all plants appeared dead.

The exceptional success of these treatments indicates that my earlier practice of brushland-to-grassland conversion can be improved. That earlier practice consisted of cutting tall brush and waiting for regrowth, which then was treated with high-volume solutions. At the present time it appears that the small stumps can be killed at any season with low-volume solutions.

It can be inferred from the over-all success of these applications that the concentrations used were higher than necessary. It is possible that solutions with concentrations as low as 5 and 10% might be effective. It should be noted that no large stumps were treated in this study.

If standing dead brush is not detrimental to the interests of the land use, then even stump treatment is superfluous and unnecessarily costly. The following two sections of this paper deal with the most efficient techniques of brush-killing: applications to trunk wounds or to the bark of the trunk base.

Applications to trunk wounds

The trunk wounds employed are of several types. Single wounds a few cm. in diameter may be made near the base of the tree with a large knife or ax; or several such wounds may be made around the trunk. "Frilling" is accomplished by downward ax blows, extending around the trunk and cutting into the wood, but without removal of chips, so that a ragged collar is formed.

Single wounds are in general unsatisfactory, as judged by treatment of about a hundred trees 7–10 m. tall, including pines, birches, aspens, maples, and others. The effects of applied herbicides generally move upward very quickly but peripherally very slowly from the wound, with the result that those parts of the tree opposite the wound remain unaffected for long periods. The fire cherry (*Prunus pensylvanica*) so treated in September, 1947 (3:250) had one lowermost branch (opposite the wound) still alive in the spring of 1949. The tree was completely dead by midsummer of 1949, apparently more from secondary fungal infections than from the herbicide. Other large and/or resistant trees give no indication of dying completely from single-wound treatments.

Frilling is far more satisfactory, as judged by treatment of approximately one hundred medium-sized trees. A Norway spruce (*Picea abies*)—a species unaffected by any of the author's previous herbicidal treatments—20 cm. in diameter was frilled March 5, 1949, and treated with 23% D-T in Sovasol no. 5. No effect was noticeable until December, 1949, when the entire tree appeared to be turning yellow.

Applications to trunk wounds may prove to be the most efficient herbicidal treatment for thick-barked standing trees. The method would be recommended also for thin-barked standing trees except that the far simpler technique of basal-bark treatment (see below) is beginning to show such excellent results

that all wounding of such plants may be entirely unnecessary.

Applications to bark of trunk bases

Such applications involve the painting, spraying, or squirting of either the lowermost 45 cm. of the shoots of woody plants, or of a 15-cm. vertical section of the shoots at a convenient height above the ground. Tests of this herbicidal technique have yielded to date results so greatly superior to all other treatments of woody plants that—until evidence appears to the contrary—all unwanted brush and smooth-barked trees will be treated in this manner at Aton Forest. The marked advantages lie in the assurance of a *one*-treatment kill, in utilizing the winter months for the work even when there is some snow on the ground, in low-volume sprays, in eliminating the danger of spray-drift damaging adjacent plants (since other plants are not in leaf, and since the spray nozzle is held close to the ground), and in eliminating labor costs of trunk-wounding, chopping, piling, and burning of brush.

Erect canes of *Rubus* were painted at the base with various solutions of 35% concentration in several of the fall and early winter months. All failed to leaf out the following spring.

Of the three white pines reported upon earlier (3:250) and treated July 12, 1948, none of the trees is dead as yet, and the lower branches of two of them appear to be growing normally. All parts above the point of application are dead. Lethal effects have moved downward only through one set of lateral branches. Failure to kill in this case is attributed entirely to the fact that the herbicide was applied high up on the trunk and not basally.

During the winter of 1948–1949, various solutions were painted or squirted on various kinds of shrubs and trees, in concentrations from 23% D-T and T (in Sovasol no. 5) to 35% (full commercial strength) D-T and T. Species treated included birches (fig. 3), staghorn sumach (*Rhus typhina*), alder, willows, aspen, red maple, northern red oak (*Quercus rubra*), sugar maple (*Acer saccharum*), white pine, white ash, and choke-cherry, with trees up to 10 m. in height and 25 cm. in diameter. Reactions were usually greatly delayed but, when they did occur, were often startling and spectacular. Of two tall adjacent aspens so treated in October of 1948, both began to leaf out and grow normally in the spring. The foliage of one, however, was completely dried and browned before the leaves had half-expanded. The other continued to grow normally through July 21 (fig. 4). On July 28 it was entirely yellow and shortly after was completely brown. A sugar maple 7 m. tall leafed out and grew normally until July 1. On July 21 it also was entirely yellow and on July 28 entirely brown. A white ash 2 m. tall did not leaf out. Pines grew variously, with the leaders more or less distorted, growth more or less limited, and herbicidal effects apparently still continuing to develop 9 months later. In general, it may be said that deciduous trees leafed out normally and grew for varying periods, when suddenly the foliage of the entire tree would turn yellow and brown—never to green again. At the inception of the 1949–1950 winter, no stump sprouts, root suckers (even from the otherwise actively suckering aspen), or other shoots had appeared from any of these trees. There were three sole exceptions among the several hundreds treated; these were three feeble maple shoots, 10 cm. tall, from as many trees, that gave little indication of survival. Bark reactions were variable.

Birches and red oak developed spongy blisters (appearing like those of balsam fir) extending several meters up the trunk. Cherries showed gummosis. Choke-cherry underwent a marked thickening of the trunk at the point of treatment, with gummosis and cracking of the bark. Alder developed light-brown, spongy thickenings over large areas. White pine showed necrotic areas extending out from the lenticels, as well as large, dark, discolored areas, and other large, light-colored, spongy areas. By early winter of 1949–1950 many of these trees were showing secondary fungal infections, and others (as white pine) still appeared in process of dying. One group of young pines treated with 23% D-T in Sovasol no. 5 in December, 1948, were stunted and yellowed in December, 1949; whereas an adjacent group of pines treated similarly with 35% T were completely dead. A small low juniper and a small red cedar, each treated with 23% D-T in

FIGS. 3, 4.—Fig. 3 (*left*), gray birch that had grown through and over high-bush blueberries, killed by painting of basal bark with 32% butyl 2,4-D on December 2, 1948. Leafed out in spring; dead by midsummer. Photographed on July 26, 1949. Fig. 4 (*right*), two trembling aspens, being killed by painting of basal bark with 35% D-T on October 12, 1948. Tree to right leafed out but browned and dried before leaves were fully expanded. Tree to left appeared normal on July 21 but had become entirely yellow when picture was taken on July 26 and was brown and dried a week later. Both grassland and background forest were open land in 1926; background was allowed to develop to forest; foreground was mowed annually through 1945; then converted to grassland with herbicides.

Sovasol no. 5 in December, 1948, were both completely dead by midsummer, 1949, except for a basal branch in each case that joined the main trunk below the points of application. Such evidence indicates that effective treatment must include the basal parts of all lower branches which may join the trunk below the point of treatment. All these tests—even if there is a percentage of resprouting in the spring of 1950—indicate a far more efficient technique of selective spot-killing than has been hitherto available, especially for such resistant plants as choke-cherry, aspen, maples, red oak, pine, juniper, and red cedar. The disadvantages of this technique lie mainly in the delayed reactions, which appear 6–12 months or longer after the application.

A different test on basal bark utilized spraying, rather than painting or squirting, of the basal 45 cm. of all stems. In this case less than 2 gallons of 20% D-T (butyl and propyl esters, respectively) in water were applied on December 2, 1948, to 150 sq. m. of dense brush 1.0–2.5 m. high. This brush included juniper, pine, fetterbush, aspen, red maple, choke-cherry, gray birch (fig. 2), hardhack, and tall blueberry. A separate test was run on striped maple (*Acer pensylvanicum*), using 5% isopropyl 2,4,5-T, applied April 10. All deciduous brush leafed out normally in the spring but browned and died before the leaves were fully expanded. Juniper and pine began to bud out, yellowed, and died by midsummer. In the autumn there was no resprouting of any species, and the kill of all woody plants in this tract appeared to be complete, without exception.

The apparent success of all sprays to basal bark, as used to date, would imply that the concentrations of sprays were stronger than necessary. Furthermore, in some cases the grass below the woody plants was affected, and locally it died. It is possible that a 10%, or even a 5%, solution would be sufficient for effective herbicidal action.

Conclusions and recommendations

1. Continued experience with over 116 species of herbs, shrubs, and trees on Aton Forest, Norfolk, Connecticut, have revealed more efficient techniques for obtaining herbicidal effects through the use of chlorophenoxy compounds diluted in water. Broad-leaved herbs (i.e., any herb other than grass) and woody plants are best treated by individual techniques.

2. Broad-leaved herbs appear to be most effectively controlled by applications in early spring. Those with winter rosettes of leaves can be sprayed before the initiation of growth. Others, such as goldenrods and asters, can be controlled best when they are still small, thus requiring a lower volume of spray to cover them. The various kinds of esters are apparently equal in their effects. There appears to be no advantage of 2,4,5-trichlorophenoxyacetates (2,4,5-T) alone over a mixture of equal parts of 2,4-dichlorophenoxyacetates and 2,4,5-trichlorophenoxyacetates. There also appears to be no advantage of a 0.5% solution over a 0.25% solution, and possibly a disadvantage, since there is some evidence that the stronger mixture rapidly burns the foliage, perhaps preventing the movement of the chemical back into other parts of the plant. Blackberries are controlled by cutting in winter and spraying the new shoots as they appear; or they may be treated as shrubs. Blackberries are sensitive to 2,4,5-T, and dense stands originally totaling about 1 acre have been completely eliminated. Some herbs (*Apocynum androsaemifolium*, *Uvularia sessilifolia*, *Solanum dulcamara*, and *Lysimachia ciliata*) may require several

sprayings to eliminate them. *Sedum purpureum* is still surviving despite 3 years of sprayings. *Asclepias syriaca* appears to be controlled with repeated stem-paintings of 35% solutions. All ferns are resistant; *Dennstaedtia punctilobula*, *Onoclea sensibilis*, and *Pteridium aquilinum* require constant attention, since on recuperation they tend to spread rapidly.

3. Woody plants have shown such striking herbicidal effects with sprays to basal bark that this efficient technique should take precedence over all others whenever possible and when the dead standing brush is not undesirable. Applications to basal bark can be made throughout the winter months and possibly through the summer. The utilization of low volumes, close to the ground, lessens the quantity of solution to be handled and the possibility of spray-drift damaging near-by plants. Solutions of 20–25% have been highly effective, and concentrations as low as 5–10% may be found to be usable. Some blackberry canes were sprayed basally in winter, none of which sprouted in spring. Because the effects of the chemical do not move downward readily in some plants, spray should be applied below the lowermost branches, or at least to the bases of the lower branches. Bark sprays are slow in their effects, and plants may leaf out and grow 5 months after the time of application, although death may occur after that. In some cases, herbicidal effects are still developing 1 year after application. Species previously unaffected —such as Norway spruce, white pine, mountain laurel, low juniper, and red cedar—now give evidence of being controllable with a single application.

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ATON FOREST
NORFOLK, CONNECTICUT

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FACTORS AFFECTING THE ACCUMULATION OF RUBBER IN SEEDLING GUAYULE PLANTS

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Introduction

Seedling guayule (*Parthenium argentatum* plants, grown either experimentally in greenhouses or in field nurseries for large-scale production, generally have a very low rubber hydrocarbon percentage—0.5–4.0% at the age of 6 months. One or two exceptions have been noted. Plants from two of the ten nurseries operated by the Emergency Rubber Project in California contained 7–8% rubber hydrocarbon as contrasted to the 2–4% found in plants from the other nurseries (13). The number of plants per square foot and the methods of culture were standardized, although the climatic and edaphic conditions of the sites of these nurseries differed. This paper describes the results obtained from various experiments conducted to ascertain the values of factors, or combinations of them, which might bring about very high rubber percentages in guayule seedlings 6 months old.

Material and methods

It has been shown that differences in temperature (1, 3), light intensity (10), season of the year (2, 7), soil moisture (2, 6), and available nutrients (4, 9) may affect the rubber percentage of older guayule plants. In the experiments described here guayule plants in half-gallon glazed crocks were grown in the greenhouses at Salinas, California, for a period of 6 months from seed under various levels of the factors mentioned above.

Two levels of temperature were maintained in separate greenhouses. The high temperature averaged 75° F. in a greenhouse thermostatically controlled so that the temperature was maintained at 70°, except during the middle of the day, when it generally rose to 85° or 90°. The low-temperature condition, averaging about 60° F., was obtained in a section of the greenhouse connected with a large evaporator and with water flowing over the glass roof during the day. The temperature in this greenhouse never rose above 75° in the daytime and reached a minimum of 45°–50° at night.

Light intensity was altered by shading the plants with cloth transmitting the designated percentage of the radiation that penetrated the one layer of greenhouse glass.

Two levels of soil moisture were maintained by growing the plants in a weighed amount of soil of a certain moisture content. A high level of soil moisture (low moisture stress) was obtained by watering daily and weighing the cultures occasionally to make certain that too much water was not being added. The low soil moisture treatment (high moisture stress) was maintained by allowing the soil to dry out until a weight was reached which indicated that the soil moisture was close to the wilting percentage, then adding sufficient water to bring it back to its original weight, and continually repeating this cycle. The time required for the soil to dry out to the desired level varied

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with the other conditions in which the plants were grown.

Available nutrients were controlled by growing the plants in sand culture and watering with the complete or minus-nitrogen nutrient solutions described by HOAGLAND and ARNON (5), including iron and minor elements. The low-nitrogen nutrient solution used was a modification of these.

The plants were grown in combinations of the different levels of soil moisture, light, and temperature or of available nitrogen, light, and temperature. Variations in soil moisture and available nitrogen were never combined in an experiment because of the doubt that arises when fertilizers are added to a soil about which little is known concerning its supply of available nitrogen or other nutrients.

At the close of the experiment the dry weights of the plants were determined by drying to constant weight at 65° C. Resin and rubber contents were determined by the turbidimetric method of TRAUB (11) and carbohydrates by the methods of TRAUB and SLATTERY (12) and MCRARY and SLATTERY (8).

Three distinct experiments were performed. In the first the plants of strain 593 were seeded on December 12, 1946, and grown for 3 months under uniform conditions of high temperature, light, soil moisture, and nitrogen, and then for 3 more months in the treatments shown in table 1.

In experiment II the seedlings of strain 4265 were placed in the differential treatments (table 1) as soon as they emerged, without allowing for a period of uniform growth. The treatments were the same as those in experiment I except that a low-nitrogen nutrient solution was used instead of the minus-nitrogen solution. The plants were seeded on March 13, 1947, and harvested on September 13, 1947.

In experiment III plants of strains 4265 and 593 were seeded on October 24, 1947, and harvested on March 30, 1948. They were grown in sand culture at the high or low temperature level and supplied with nutrient solution containing high or low nitrogen. These plants were also given a uniform start of 6 weeks at high-temperature, high-nitrogen levels.

TABLE 1

DIFFERENTIAL TREATMENTS USED IN EXPERIMENTS I AND II

F.	
75°	high soil moisture, 100% greenhouse light
75°	low soil moisture, 100% greenhouse light
75°	high soil moisture, 60% greenhouse light
75°	low soil moisture, 60% greenhouse light
60°	high soil moisture, 100% greenhouse light
60°	low soil moisture, 100% greenhouse light
60°	high soil moisture, 60% greenhouse light
60°	low soil moisture, 60% greenhouse light
75°	high-nitrogen nutrient solution, 100% greenhouse light
75°	minus-nitrogen nutrient solution,* 100% greenhouse light
75°	high-nitrogen nutrient solution, 60% greenhouse light
75°	minus-nitrogen nutrient solution,* 60% greenhouse light
60°	high-nitrogen nutrient solution, 100% greenhouse light
60°	minus-nitrogen nutrient solution,* 100% greenhouse light
60°	high-nitrogen nutrient solution, 60% greenhouse light
60°	minus-nitrogen nutrient solution,* 60% greenhouse light

* In experiment II a low-nitrogen nutrient solution was used instead.

Every treatment in each experiment contained twenty-five plants, divided into five replications of five plants each. In harvesting, the five plants of each replication were combined for the various determinations. Chemical analyses were made on each replication. Analyses of variance were carried out, and, where

significant differences were shown to exist, the difference required for significance at the 5% level was calculated.

Results

In presenting the results, only the data from experiments II and III have been tabulated. The results obtained in experiment I were so similar to those of experiment II that their tabulation would only make for unnecessary duplication, even though different strains were grown. Occasionally data from experiment I have been cited in the text to emphasize certain points.

DRY WEIGHT.—Plants grown on the low levels of light intensity, temperature, soil moisture, or available nitrogen supply generally showed lower dry weights of leaves, stems, and roots than those on the high levels, if the other conditions were held constant (table 2). The exceptions to this were found in the dry weights of roots; in the soil cultures the low levels of available soil moisture or temperature often had no effect on root weight in comparison with that of the plants on the high levels. For plants grown at the different temperatures, the roots showed the least difference in dry weight and the stems the greatest. Different levels of soil moisture and available nitrogen likewise had less effect on dry weight of roots than on that of tops. Shade, however, produced a converse effect.

The relative effects of the levels of the various factors on growth were estimated by calculating from the data of experiments I and II the percentage reduction in dry weight when the level of one factor was changed while the others were maintained constant. Thus, under the conditions of these experiments, the difference in temperature had the greatest effect on the dry weight of leaves, while the effects

TABLE 2

DRY WEIGHT PER PLANT (GM.) OF GUAYULE SEEDLINGS GROWN IN DIFFERENT ENVIRONMENTAL CONDITIONS

ENVIRONMENTAL CONDITIONS	EXPERIMENT II*—STRAIN 4265			
	100% light intensity		60% light intensity	
	75° F.	60° F.	75° F.	60° F.
Leaves:				
High soil moisture	6.57	5.23	3.73	1.69
Low soil moisture	3.54	2.34	1.61	1.32
High available nitrogen	7.50	6.49	4.76	2.45
Low available nitrogen	4.10	2.78	2.56	1.28
Stems:				
High soil moisture	2.33	1.59	1.53	0.51
Low soil moisture	1.33	0.67	0.58	0.36
High available nitrogen	3.95	2.37	2.17	0.78
Low available nitrogen	1.33	0.72	1.22	0.39
Roots:				
High soil moisture	2.71	2.74	0.72	0.42
Low soil moisture	1.81	1.50	0.35	0.50
High available nitrogen	4.45	3.45	1.09	0.59
Low available nitrogen	2.30	1.83	0.66	0.37

	EXPERIMENT III†—100% LIGHT INTENSITY			
	Strain 593		Strain 4265	
	75° F.	60° F.	75° F.	60° F.
Leaves:				
High available nitrogen	6.80	2.46	6.99	2.77
Low available nitrogen	3.20	1.38	3.61	1.32
Stems:				
High available nitrogen	3.50	0.48	2.82	0.45
Low available nitrogen	1.03	0.27	1.08	0.21
Roots:				
High available nitrogen	3.73	1.03	3.83	1.18
Low available nitrogen	1.90	0.77	2.44	0.74

* Least significant difference $P < 0.05$; leaves 0.67, stems 0.24, roots 0.24 gm.

† Least significant difference $P < 0.05$; leaves 0.99, stems 0.21, roots 0.57 gm.

of the different levels of the other factors were about equal but less than that of the difference in temperature. Changes in levels of each factor affected the dry weight of stems to about the same degree. The rank of factors in the relative effects of their levels on the dry weight of roots, arranged in descending order, were: first, light intensity; second, available nitrogen; third, soil moisture; and, fourth, temperature. Thus, as an average, the dry weight of roots was about 75% less on low light intensity than on high, while the difference in root dry weight at the two temperature levels was about 20%. The effect of different levels of temperature and light intensity seemed to be greater for plants in sand culture than for those in soil. This may indicate an interaction between soil moisture and nitrogen supply so far as the effect of temperature is concerned.

In experiment III no difference in response to the levels of the different factors was noted between strains 4265 and 593. There was likewise no indication that one strain grows more vigorously than the other.

CARBOHYDRATE CONTENT.—No significant differences with treatment were observed in the free-sugar or inulin content of the plants. For example, the levulin percentage in the stems of the plants in experiment II (table 3) varied from 0.88 to 9.35, while the sugar and inulin contents were always less than 1.00. For this reason, the results of the sugar and inulin determinations are not shown.

The levulin content varied markedly with the conditions under which the plants were grown (table 3). In plants grown in the high light intensity the low levels of temperature, soil moisture, or available nitrogen always resulted in higher percentages of levulins in the stems and roots than did the high levels of these factors. In the low light intensity, however, the levulin contents of comparable plants were lower than in high light intensity. The effect of differences in levels of the other factors was also much less when the plants were growing under the shades. In experiment III, both strains showed the same response to levels of temperature and nitrogen supply. The only difference noted be-

TABLE 3

LEVULIN PERCENTAGE OF GUAYULE SEEDLINGS GROWN IN DIFFERENT ENVIRONMENTAL CONDITIONS

Environmental conditions	Experiment II*—Strain 4265			
	100% light intensity		60% light intensity	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High soil moisture	2.09	6.54	0.88	1.44
Low soil moisture	2.83	8.07	1.40	2.20
High available nitrogen	1.10	5.84	1.40	1.20
Low available nitrogen	2.92	9.35	3.52	3.46
Roots:				
High soil moisture	3.46	4.95	2.22	2.21
Low soil moisture	5.46	7.53	2.74	4.06
High available nitrogen	2.64	7.76	2.47	2.47
Low available nitrogen	6.19	11.70	8.31	8.16

	Experiment III*—100% light intensity			
	Strain 5934		Strain 4265	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High available nitrogen	4.06	15.42	2.52	14.56
Low available nitrogen	3.05	21.64	3.13	19.81
Roots:				
High available nitrogen	1.84	3.12	4.78	7.87
Low available nitrogen	4.78	7.52	7.12	12.76

* Least significant difference $P < 0.05$ for experiment II, 0.88%; for experiment III, 1.07%.

tween the strains was the higher percentage of levulins in the roots of 4265 than of 593.

TABLE 4

RESIN PERCENTAGE OF GUAYULE SEEDLINGS GROWN IN DIFFERENT ENVIRONMENTAL CONDITIONS

Environmental conditions	Experiment II*—Strain 4265			
	100% light intensity		60% light intensity	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High soil moisture	9.51	7.69	4.22	3.31
Low soil moisture	8.95	8.33	4.41	4.42
High available nitrogen	9.73	7.03	4.26	3.49
Low available nitrogen	13.75	9.97	5.57	4.40
Roots:				
High soil moisture	5.79	4.78	2.64	1.90
Low soil moisture	4.42	4.60	2.00	2.00
High available nitrogen	5.10	3.36	1.93	1.86
Low available nitrogen	5.96	4.21	2.73	2.43

	Experiment III†—100% light intensity			
	Strain 593		Strain 4265	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High available nitrogen	3.82	3.88	6.93	5.93
Low available nitrogen	4.31	4.11	9.10	6.87
Roots:				
High available nitrogen	1.46	1.92	2.74	2.51
Low available nitrogen	2.34	2.20	4.29	2.86

* Least significant difference $P < 0.05$; stems 0.90%, roots 0.43%.

† Least significant difference $P < 0.05$; stems 0.89%, roots 0.50%.

Taken as a whole, the difference in temperature had the greatest influence on the levulin content of the plants, followed by the difference in light intensity, with those of soil moisture and available nitrogen having the least, although a very pronounced, effect.

The pentosan content averaged about 19% for the stems and 15% for the roots in all the treatments. There was no indication that the pentosan content was affected by the range in conditions to which the plants were exposed; for this reason, the results of these determinations are not shown.

RESIN CONTENT.—Plants on high light intensity always had a higher resin percentage than those on the low level (table 4). The low levels of soil moisture and available nitrogen, however, usually produced a higher resin percentage in the plants than did the high levels. Plants of strain 4265 had a higher resin percentage on high than on low temperature, but no consistent difference was noted between the plants of strain 593 on the different levels of this factor.

As indicated by the results of experiment III, the plants of strain 4265 had a much higher resin percentage than those of strain 593. The very high resin percentage of the stems in the treatment combining high light intensity and high temperature and low available nitrogen in experiment II should be noted.

Although these experiments were carried out primarily to emphasize the percentage rather than the gram content of resins in the plants, the latter values were calculated. The results (not presented) indicated that the grams of resin per plant were more closely associated with the growth of the plant or its dry weight than with the percentage content. In other words, the biggest plants yielded the largest amount of resin, regardless of their percentage.

RUBBER CONTENT.—The degree of shading employed so greatly limited the rubber content of the plants at the lower light intensity that no effect of the dif-

ferent levels in the other factors was apparent (table 5). The remaining discussion under this heading applies only to the plants grown at the high light intensity.

The plants on low soil moisture always had a higher percentage of rubber than those on the high level. The stems of the plants grown in soil showed a higher rubber percentage on the low than on high temperature, but no significant effect of temperature level was observed on the rubber percentage of the roots. When the plants were grown in nutrient solutions, however, both stems and roots showed a higher rubber percentage on high than on low temperature. In other words, there was an interaction between culture types and temperature levels as far as their effect on the rubber percentage of the plants was concerned. This unexpected effect of high temperature producing a higher percentage of rubber than low temperature was also noted for the plants of experiment I in sand culture. Stems of plants on low available nitrogen usually showed a higher percentage of rubber than those on high nitrogen, but the roots showed no significant response of their percentage of rubber to different levels of available nitrogen. No consistent differences were noted between the rubber contents of the two strains.

The relative effectiveness of the differences between the levels of the various factors in altering rubber percentage, arranged in descending order, were: first, light intensity; second, temperature; third, soil moisture; and, fourth, available nitrogen.

The grams of rubber per plant are shown in table 6. As was true of the resin, the yield of rubber was for the most part correlated with the dry weight of the plants rather than with the percentage of rubber. In a few instances a set of conditions which produced a plant of a lower dry weight than another set of conditions also produced a much higher percentage of rubber so that the smaller plants showed the greater yields. The large yield of rubber in plants in the treatment combining high light intensity, high nitrogen, and high temperature is worthy of note.

TABLE 5

RUBBER PERCENTAGE OF GUAYULE SEEDLINGS GROWN IN DIFFERENT ENVIRONMENTAL CONDITIONS

Environmental conditions	Experiment II*—Strain 4265			
	100% light intensity		60% light intensity	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High soil moisture	1.20	1.64	0.18	0.09
Low soil moisture	1.96	2.76	.20	.15
High available nitrogen	2.77	1.16	.27	.07
Low available nitrogen	3.13	1.71	.46	.13
Roots:				
High soil moisture	0.74	0.55	.15	.13
Low soil moisture	0.77	0.73	.11	.16
High available nitrogen	1.53	0.61	.23	.18
Low available nitrogen	1.22	0.66	0.33	0.29

	Experiment III†—100% light intensity			
	Strain 593		Strain 4265	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High available nitrogen	1.63	0.25	1.40	0.24
Low available nitrogen	1.80	.49	1.45	.53
Roots:				
High available nitrogen	0.79	.12	1.65	.10
Low available nitrogen	0.97	0.10	0.62	0.11

* Least significant difference $P < 0.05$; stems 0.29, roots 0.24%.

† Least significant difference $P < 0.05$; stems 0.34, roots 0.13%.

In both sand and soil culture the plants yielded less rubber on the low than on the high temperature level; however, this reduction in yield was much greater for the plants in sand culture. This is related to the different effects of temperature level on the rubber percentage of plants growing in the different media. The yield of rubber per plant was higher in strain 593 than in 4265.

TABLE 6

GRAMS OF RUBBER PER PLANT IN GUAYULE SEEDLINGS GROWN IN DIFFERENT ENVIRONMENTAL CONDITIONS

Environmental conditions	Experiment II*—Strain 4265			
	100% light intensity		60% light intensity	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High soil moisture	0.028	0.026	0.003	†
Low soil moisture	.026	.019	.001	†
High available nitrogen	.100	.024	.006	0.001
Low available nitrogen	.041	.012	.006	.001
Roots:				
High soil moisture	.020	.015	.001	†
Low soil moisture	.014	.011	†	.001
High available nitrogen	.068	.021	.002	.001
Low available nitrogen	0.028	0.012	0.002	0.001

	Experiment III‡—100% light intensity			
	Strain 593		Strain 4265	
	75° F.	60° F.	75° F.	60° F.
Stems:				
High available nitrogen	0.056	0.001	0.041	0.001
Low available nitrogen	.019	.001	.014	.001
Roots:				
High available nitrogen	.029	.001	.025	.001
Low available nitrogen	0.018	0.001	0.014	0.001

* Least significant difference $P < 0.05$; stems 0.006, roots 0.005 gm.

† Values less than 0.0005 gm.

‡ Least significant difference $P < 0.05$; stems 0.015, roots 0.007 gm.

Under the conditions studied, the difference between the levels of light intensity produced the greatest differences in rubber yield, followed in turn by differences in the levels of temperature, then available nitrogen in the sand cultures and by soil moisture, then temperature in the soil cultures.

Discussion

The results indicate fairly well the effect of the various combinations of levels of environmental conditions on the growth and chemical composition of guayule seedlings. Plants grown at the low level of light intensity always showed less dry weight, rubber and resin percentages, and usually less levulin percentage than those grown at the high level. In some treatments the low light intensity was the limiting factor, so that any effect of levels of other factors was not apparent. For this reason, unless otherwise stated, the discussion of the effects of levels of the other factors will deal only with their effects in the high light intensity.

With one or two notable exceptions, any variation in soil moisture, available nitrogen, and temperature which resulted in lower dry weight also resulted in higher percentage composition of levulins, resins, and rubber.

From a qualitative standpoint, the lower the dry weight, the higher the rubber percentage; but this inverse relationship was not proportional. When the levels of two factors tending toward low dry weight were combined in a single treatment, the resulting dry weight was lower and the percentage composition was higher than that produced under the same level of either factor combined with the other level of the other factor. The

growth response of the plants resulting from the combination of the low growth-producing levels of two factors in a single treatment could not be estimated from the growth responses to the low level of each factor in two different treatments.

One notable exception to the general observation that plants with lower dry weights had a higher rubber percentage than those with the greater dry weights was found in the sand-culture series. Plants grown at 60° F. had a lower dry weight than those grown at 75° F., but they also had a lower resin and rubber content. In other words, the plants on high temperature showed both a greater dry weight and a greater percentage of rubber than those grown at the low level. This response occurred in all three experiments at the high light intensity, regardless of the strain of guayule and, with only one exception, regardless of the level of available nitrogen. In these same experiments plants grown on low available nitrogen showed the same or a slightly greater rubber percentage than those grown on the high level. Where direct comparisons between soil and nutrient culture were possible, the rubber percentage (calculated from the root and stem values) of the entire plants (less leaves) grown in the treatment combining high temperature, high nitrogen, and high light intensity was higher than any percentage reached in soil cultures. The dry weight (less leaves) of the plants in this treatment in experiment II was 44% greater than in any other sand culture and 67% greater than in any soil treatment; even greater differences occurred in experiment I.

Here, then, is a case where the largest plants had, if not the highest, at least the next to the highest rubber percentage of plants grown in sixteen different treatments. Thus, a high percentage of rubber is not incompatible with a large growing plant. When the plants were grown in a combination of levels of different factors which tended to produce a high percentage of rubber and a large dry weight, the rubber yield was greatly increased. For example, the plants grown on the high levels of light intensity, temperature, and available nitrogen yielded 2.75 times as many grams of rubber per plant as those in any other treatment in experiment I.

Although, in these experiments, the rubber content of plants (less leaves) did not reach 8%, approximately the highest percentages occurred in the plants which were growing in sand culture at a fairly high mean temperature and receiving abundant moisture and nitrogen. The sand used in these cultures, although acid washed, was obtained from the dunes on the Monterey Peninsula. It is interesting that the nurseries, mentioned in the introduction, in which large seedlings with 7–8% of rubber were produced, were located on very sandy soil along the Pacific Coast near Oceanside, California. These nurseries were heavily watered and regularly fertilized and so had ample available supplies of nitrogen.

This effect of temperature level on the rubber content of the plants in sand culture is contrary to the usual effect of temperature level but has always occurred when seedlings were grown under such conditions in the greenhouse at Salinas. No explanation for this can be offered at present. The fact that the levulin content of the plants was higher at the low temperature than at the high in both soil and sand cultures, which is usual, eliminates the possibility of any complete disruption of the normal metabolism of the plants in this type of culture. It is possible that such a temperature effect occurs only with seedling plants and not with the plants grown from nursery stock on

which previous experiments were conducted.

These results show that plants growing at lower temperatures are not necessarily forming rubber faster than those at a higher temperature, on either a percentage or a grams-per-plant basis.

The results also show that the processes by which the various compounds studied are produced are more or less distinct from one another. For example, the pentosan content remained constant regardless of the conditions under which the plants were grown. Temperature level had a greater effect on the levulin percentage than on the rubber and resin percentages. In several instances plants grown at one temperature level showed a higher rubber percentage and lower resin percentage than those grown at the other level. Soil moisture level had a greater effect on the rubber than on the resin content. For example, in experiment II plants grown at low soil moisture showed a higher percentage of rubber and about the same percentage of resin as those grown at the high level. The resin and rubber contents parallel each other more closely, however, than any of the other constituents determined. In other words, the percentages and even the grams per plant of the various compounds vary independently of one another in the different conditions. To carry this idea further, the grams of rubber formed were divided by the grams of each of the other compounds determined in the hope that a constant quotient would be obtained for one of the ratios. This would indicate that, for each gram of a certain compound formed, a given number of grams of rubber would be formed—or that rubber formation was related to the formation of the other compound. No such constant quotient was obtained.

Of the compounds studied, the levulin percentage showed the greatest variation with treatment, followed in turn by the percentages of rubber, resin, sugar, inulin, and pentosans.

The only consistent difference between strain 593 and 4265 was the much higher resin content of the latter. Reports (**14**) have shown that older plants of strain 4265 grow more vigorously and have a higher rubber percentage than those of strain 593. No evidence of such differences was apparent in these experiments. Except in the resin content, no difference in kind of response to the various treatments was observed between the two strains.

Summary

1. Guayule seedlings of strains 593 and 4265 were grown to an age of about 6 months in two levels of light intensity, soil moisture, temperature, and available nitrogen and various combinations of these conditions. The dry weights, sugars, levulins, inulin, pentosan, resins, and rubber content of the plants were determined.

2. Dry weight of plants and percentages of determined constituents were generally higher on the high than on the low level of light intensity.

3. At high light intensity, plants grown at the high levels of temperature, soil moisture, and available nitrogen always showed a greater dry weight and, with one notable exception, a lower percentage of levulin, resins, and rubber than those grown at the low levels of these factors. The exception was the higher rubber percentage of the plants grown at high temperature in sand culture than of those grown at the low level.

4. Plants grown in sand culture at the high levels of available nitrogen, temperature, and light had the greatest dry weight (less leaves). Because of the re-

sponses of the plants in sand culture to different levels of temperature and available nitrogen, these plants had the next to the highest rubber percentage of those from sixteen treatments and higher than any plants from the soil cultures. High rubber percentage is thus not incompatible with rapid growth under some conditions. For total rubber produced, these plants outyielded those on any other treatments by better than two and one-half times.

5. There was no observable interrelationship among the compounds as far as the response of their percentage content to the different levels of the various factors was concerned. This would seem to indicate that the various compounds are formed more or less independently of one another.

6. No evidence was obtained in these tests that seedlings of strain 4265 grow more rapidly or produce more rubber than do those of strain 593.

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ON THE INHERITANCE OF APOMIXIS IN PARTHENIUM ARGENTATUM

D. U. GERSTEL AND WM. MISHANEC[1]

Introduction

Guayule (*Parthenium argentatum* Gray) is part of an apomictic complex (**16**) in which the diploid ($2n = 36$) races reproduce sexually, but the polyploids with 54 or more chromosomes are facultative apomicts (**3, 16, 22**).[2] This distinction is quite general in the wild; under cultivation apomictic "haploid" plants with approximately the diploid number of chromosomes can survive. These may arise from tetraploids by the parthenogenetic development of reduced eggs; a great number of such polyhaploids could be secured by selection at an early seedling stage, using stomate measurements, and by careful nursing of the selected plantlets. Some tetraploids apparently produce more polyhaploids than others (MISHANEC, unpublished). It goes without saying that the genetical analysis of apomixis is made considerably easier by the existence within a species of apomictic plants having the same chromosome number as sexuals. CATCHESIDE (**1**) has suggested a procedure for the utilization of these plants in the analysis of the inheritance of apomixis, and his recommendations were followed to a considerable extent in the present investigation.

Material and methods

The polyhaploid used as a parent (42354-P) had been detected by Dr. R. C. ROLLINS among plants from a Mexican collection made in 1942 by Dr. LEROY POWERS. It was chosen because of its relative fertility, fair vigor, and profuse flowering capacity. Reduction of the chromosome number from 73 in the parental strain to 37 in this plant had not eliminated the apomictic mode of reproduction (facultative apomixis); a part of its seed progeny consisted of plants resembling the mother in chromosome number and in morphology. Especially characteristic were the narrow leaves and prostrate habit (fig. 1). It could be assumed that those progeny plants from 42354-P which resembled the mother in all discernible features were her accurate replicas which had arisen by unreduced apomixis. Some of the data presented below were obtained from these maternal progeny plants instead of from the mother.

Crossings were performed without emasculation unless the contrary is indicated; self-incompatibility had been demonstrated before in diploid guayule, including the particular diploid (SP-14) used as female in the current study (**6, 16**) and in tetraploids produced by colchicine treatment from diploids (**6, 7**). It will be shown in the following that the polyhaploid and the F_1 plants were also self-incompatible.

Hybridizations between the polyhaploid and its diploid mates were performed in an isolation plot at Stanford University. SP-14 was mated with the polyhaploid in 1947, and SP-2 in 1948.

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[2] Apomictic reproduction by generative apospory and pseudogamy in the terminology of STEBBINS (**20**), by diplospory and pseudogamy in that of GUSTAFSSON (**8**).

Interspecific crosses were made in a greenhouse at the United States Natural Rubber Research Station under isolation bags. F_2 seed from the cross sexual × apomictic were obtained in an isolation plot near Salinas as noted later in detail.

Chromosome preparations were fixed according to a schedule adapted by CATCHESIDE (1) for use with guayule. Flower buds were fixed in four parts of chloroform : three parts of absolute ethyl alcohol : one part of glacial acetic or propionic acid. The materials thus fixed were preserved in the fixative placed in an icebox, often for several weeks. Before staining, anthers were dissected in 45% propionic acid. After the acid was blotted off, they were macerated in a drop of a 1:10 dilution of concentrated hydrochloric acid, which was soaked up with bibulous paper after 2 minutes, followed by rinsing with distilled water. They were then stained in 60% propionic acid saturated with orcein and covered (staining schedules by the junior author).

Pollen diameters were measured in acetocarmine from the base of a spine of the exine to the base of an opposite spine. They averaged 16.8 μ for diploids, 19.3 μ for triploids, and 21.3 μ for tetraploids. Measurements of stomatal guard cells were resorted to where no blossoms could be obtained; these averaged (in cases where the chromosome counts had been ascertained otherwise) 22.1 μ, 28.2 μ, and 33.5 μ in length for $2n$, $3n$, and $4n$, respectively, for specimens taken from the center of mature leaves.

Observations

THE PARENTS

The sexual parent was in every respect a typical representative of diploid guayule. It reproduced sexually (to be demonstrated below), was self-incompatible, its chromosomes conjugated consistently in 18 pairs (fig. 2), its pollen production was abundant, and pollen and seed were highly fertile (table 1).

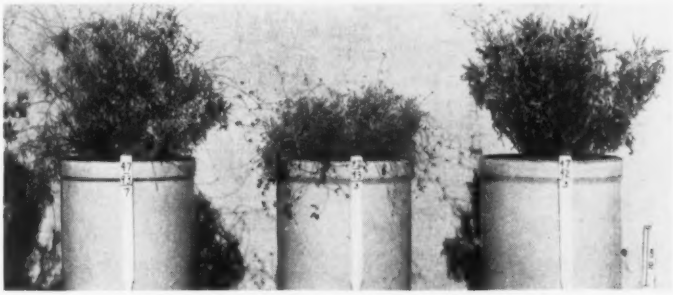


FIG. 1.—*Parthenium argentatum*. Three progeny plants of polyhaploid. Center plant has 37 chromosomes, and plant to left has 54±; both came from cross apomict × sexual. Plant on right, from reciprocal cross, has 36 chromosomes.

The apomictic type differed in more than its reproductive habit from the normal diploid; it possessed an additional, satellited chromosome—a total of 37 (fig. 3). Chiasma frequency per cell was lower than in the diploid (table 1); and pairing ranged from $11_{II} + 1_{5I}$ to $18_{II} + 1_{I}$ (fig. 4). Occasionally the extra chromosome could be observed in conjugation with a pair. Pollen was scarce; male and female fertilities were much reduced (table 1) but were high enough to produce sizable progenies.

The data in table 2 indicate that the apomict and its maternal offspring were self-incompatible: no seed were set under isolation, but, after outcrossing, 3.6% fertile seed were found.

Considering the origin of this type by haploid pseudogamy from a tetraploid parent, its abnormal reproductive habit is not surprising. The type compares favorably in its fertility with a similar "haploid" from tetraploid guayule mentioned by STEBBINS and KODANI (**21**) which set no seed at all. Other fertile polyhaploids were described by MÜNTZING (**12**) in *Poa pratensis* and by ELLIOT and WILSIE (**2**) in *Bromus inermis*. KIELLANDER (**11**) found even a "subhaploid" with 18 chromosomes in the progeny of a *Poa pratensis* with 72± chromosomes which was somewhat fertile.

CROSSES APOMICT ♀ × SEXUAL ♂

Seed collected from the apomictic partners of the pairs isolated at Stanford produced plants which could be separated without effort into "maternals" and "nonmaternals" (table 3, *a*). The maternals had the prostrate habit, slender peduncles, narrow leaves, and smaller size of the mother plant (fig. 1). In addition to the morphological classification, pollen diameters measured for most of the plants in this group also indicated diploidy. Chromosome counts made for five plants gave 37 chromosomes in each instance (the chromosome number of the mother).

The nonmaternals were all erect and had a "typical guayule" habit. They were diploids, triploids, tetraploids, and one was approximately a hexaploid, as indicated by pollen diameters. Pollen-mother-cell chromosomes of a few plants in each group were also counted and confirmed the existence of the four groups. The four diploids differed from the maternal type not only by their upright habit and other morphological features but also by their greater pollen fertility; they must have resulted from the fertilization of reduced ovules of the apomict. The 24 triploids did not differ appreciably from the nonmaternal diploids, but their larger pollen and chromosome

TABLE 1

THE PARENTS

Plant no.	Reproduction	Somatic chromosome no.	Mean no. chiasmata per cell at diakinesis (no. cells)	% Normal pollen (no. grains)	% Filled achenes (no. achs.)
SP-14	Sexual	36	25.4 (16)	99.0 (1847)	75.5 (200)
42354-P	Apomictic	37	20.3* (16)	22.1* (366)	3.6* (1960)

* Maternal type progeny plants of 42354-P substituted.

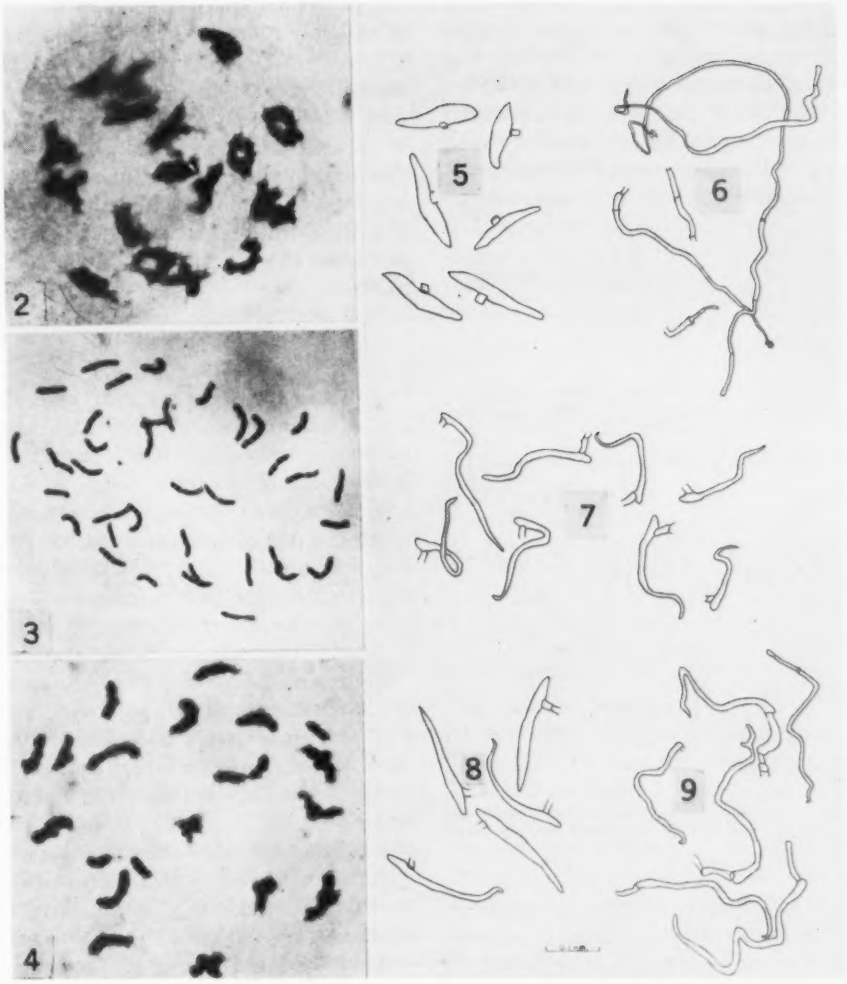
TABLE 2

SEED FERTILITY OF APOMICTIC PARENT AND OF TWO PROGENY PLANTS OF MATERNAL TYPE AFTER SELFING AND OUTCROSSING

Plant no.	Selfed seed set* (total)	Outcrossed seed set† (total)
42354-P	0 (1295)	63 (1200)
47-93-1	0 (395)	4 (490)
47-93-2	0 (1300)	3 (200)
Total	0 (2990)	70 (1960)

* Plants were isolated at three locations in central California in early summer, 1948.

† Unrelated partners were placed next to the isolates in second half of summer, 1948.



FIGS. 2–9.—*Parthenium argentatum*. Fig. 2, sexual parent: diakinesis with 18 pairs. *Ca.* ×1850. Fig. 3, apomict (37 chromosomes): somatic metaphase in tapetal cell—note three satellites. *Ca.* ×1350. Fig. 4, apomict: diakinesis with $18_{II} + 1_{I}$. *Ca.* ×1850. Figs. 5–9, trichomes. Fig. 5, pure guayule; fig. 6, pure *P. stramonium;* fig. 7, diploid guayule-*stramonium;* fig. 8, triploid guayule-guayule-*stramonium;* fig. 9, triploid guayule-*stramonium-stramonium*. (Figs. 2–4, photomicrographs; figs. 5–9. Camera lucida drawings.)

counts of ten of the group served to identify them.

Four plants had 72± chromosomes, and one even had over 100. As these high numbers occurred only in one of the four replications of that particular cross, contamination may be suspected, possibly from the seeding medium. Further experimentation would be needed to establish with certainty whether formation of ovules with twice the maternal somatic chromosome number (or more) occurs in guayule apomicts as has been described for *Potentilla collina* (13), but this question is not pertinent to the problem here.

It may be noted that one row in table 3 represents an interspecific cross, which, like the intraspecific crosses, gave maternal and nonmaternal hybrid offspring. No haploids with 18 chromosomes which could have arisen through reduction and nonfertilization were found.

TABLE 3

PROGENIES FROM RECIPROCAL CROSSES BETWEEN APOMICT 42354-P AND SEXUAL DIPLOIDS

Parents	$2n$* maternal	$2n$ nonmaternal	$3n$	$4n$ or over	Total
	a) Apomict ♀ × sexual ♂				
42354-P × SP-14	30	3	21	5	59
42354-P × SP-2	14	1	2	.....	17
42354-P × *P. stramonium*	1	.....	1†	.....	2
Total	45	4	24	5	78
	b) Sexual ♀ × apomict ♂				
SP-14 × 42354-P	.....	21	.....	.....	21

* $n = 18$; levels of polyploidy are indicated; actual chromosome numbers may have deviated slightly from the euploid numbers.

† Interspecific hybrid.

Cross sexual ♀ × apomict ♂

The chromosome numbers of thirteen of the twenty-one plants resulting from the reciprocal cross SP-14 × 42354-P were determined and found to be 36 in each case (table 3, *b*). The chromosomes of the remaining eight plants were not counted, but the habit, leaf shape, and leaf thickness indicated that these plants also were diploids which had originated as a result of the fertilization of a reduced ovule by a reduced pollen grain.

Interspecific hybridization of diploid F_1 plants

Powers and Rollins (16) were the first to use interspecific hybridization between guayule (as the female) and a related species (*P. incanum* H.B.K. in their case) for a determination of the extent of apomictic reproduction of a guayule parent. The present authors have adopted this clean-cut method in order to determine whether apomixis occurred in the F_1 plants (table 4).

Fourteen different diploid F_1 (sexual × apomict) sibs were pollinated with *P. stramonium* Greene and yielded a total of 738 seedlings (table 4, *a*). Many of these died at various stages of maturity, leaving a total of 527 for classification. Similarly, two diploid F_1 plants of the cross apomict × sexual were crossed with *P. stramonium*, yielding 75 germinants, of which 48 survived (table 4, *b*).

All 575 plants (table 4, *a*, *b*) were first analyzed for hybridity. Hybrids of guayule and *P. stramonium* are intermediate between their parents in several key characters, especially those of the leaf, as the illustrations of Rollins (18) and Tysdal (22) show. There was no difficulty in recognizing all 575 plants as interspecific hybrids; i.e., all the ovules from which they came had been fertilized by *P. stramonium* pollen.

It remained to be seen from their chromosome numbers whether these hybrids had originated from reduced or unreduced ovules. For this purpose the material was separated into two groups: (*a*) Plants which appeared to be truly intermediate diploids. As the number was large (527), it was not feasible to verify chromosome counts cytologically, so the pollen of every thirtieth of this group found in the field was measured. These measurements of pollen diameters indicated diploidy in every case; further verification was given by chromosome counts of 36 or 37 for pollen mother cells from several plants. (*b*) Plants which, in some way, differed from "typical" intermediate diploids; either their leaves were thickened, or their growth dwarfed, or they tended morphologically more toward one or the other parent (48 plants). By means of pollen measurements, or guard-cell measurements of those which had failed to bloom by the end of the 1949 season, it was found that they also were all diploids, with the exception of eight which were suspect of deviating chromosome numbers. Chromosome counts of pollen mother cells of the latter showed that five were diploids with 36 or 37 chromosomes, one was a diploid with 34 chromosomes, and two were triploids with approximately 54 chromosomes. The trichomes of these two triploids gave conclusive evidence as to their derivation (see [17] for the use of trichomes as an indicator of hybridity in guayule-mariola hybrids). One (fig. 9) had trichomes which were longer and thus more *stramonium*-like than those of the diploid hybrids (fig. 7). This plant, therefore, had originated as a result of the fertilization of a reduced guayule ovule by unreduced *P. stramonium* pollen. The trichomes of the other triploid (fig. 8) were shorter than those of the intermediate hybrids and thus resembled those of guayule more closely. This plant, consequently, had been produced by an unreduced ovule—the only plant in the population of 575 which had originated in this fashion.

TABLE 4

PROGENIES FROM CROSSES WITH P. STRAMONIUM

Mother plants		Total achenes sown	Germinants	Classified survivors	Morphology		Chromosomes		
Origin	Chromosomes				Hybrids	Guayule	2*n*	3*n*	4*n*
a) Sexual×apomict	2*n*	2147	738	527	527	0	525	2	0
b) Apomict×sexual	2*n*	296	75	48	48	0	48	0	0
c) Apomict×sexual	3*n*	462	91	58	7	51	....	....	....
d) Artificial tetraploids	4*n*	407	157	120	119*	1	0	109	2

* Nine of these hybrids died before their chromosome numbers could be ascertained. Of the two 4*n* plants, one was a hybrid, the other the pure guayule listed.

F_2 SEXUAL × APOMICT

If apomixis is controlled by a small number of genes, the segregation in the F_2 generation should provide the evidence. It should also show whether the components of apomixis[3] are genetically

[3] These are nonreduction and parthenogenesis. The necessity for endosperm fertilization in some apomicts but not in others (**8**:33) demonstrates that endosperm development must be provided for independently, a fact which has been widely overlooked (for an exception see [15]). For pseudogamous

independent. Such an F_2 population has not yet matured, but it might be well to describe here the difficulties standing in the way of its production.

In order to find sibs among the F_1 plants which were compatible, the pollen-tube method of detection of compatibility (**7**) was used in testing twenty sibs and gave the negative results shown in table 5. Although only a relatively small number of all the possible cross-combinations and crosses with both parents were tested, none of these indicated complete compatibility. A few (−?, +? in the table) produced a small number of tubes. The results from tests with unrelated plants, also included in table 5, indicated that incompatibility and not sterility was at the basis of this behavior.

The earlier study (**6**) of self-incompatibility in guayule did not reveal such a degree of incompatibility among members of a family. It would be premature indeed to speculate at this time as to what sort of situation could prevail in the present material, the complex origin of which should be remembered. It is not impossible that further pollen-tube tests would finally have revealed a compatible pair of sibs or a progeny plant capable of mating with the recessive parent. Rather than laboriously adding to the tests already performed it was decided, upon the suggestion of Dr. H. M. TYSDAL, to isolate a stand of a larger number of F_1

TABLE 5

INCOMPATIBILITIES AND COMPATIBILITIES OF F_1 SIBS FROM CROSS SEXUAL × APOMICT

PLANT	♂ SEXUAL PARENT	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	UNRELATED MALE
Sexual parent			−	−					−							−	−?		−	−		
1	−																			−		
2																				−		
3																	−					
4																	−					+
5																						
6																						
7																						
8	−																	−				+
9											−						−	−		−		+
♀ 10		−								−							−	−				
11															−		−	−				
12																						
13	−			−											−		−					
14												−		−			−		−			
15	−																	+?				
16	−			−	−				−	−	−	−		−	−			−	−	−		
17									−	+?	−						−		−	−		
18	−								−	−		−					−					
19	−	+?	−							−							−	−				
20																						
Unrelated female									+	+					+			+		+		

guayule it still remains to be shown whether endosperm fertilization occurs or the pollen stimulates development by other means.

plants together and thus to give them a chance for extensive cross-pollination. Any compatibilities, yet undetected, or mutations of incompatibility factors would then come into play and produce F_2 seed. Of course, a drawback of the method would be the masking of the fathers.

Fifteen sibs were thus isolated in a side valley of the Salinas and seed harvested from them. A low percentage of seedlings has recently been produced; the paucity (0.0%–3.0%) of germination indicated that no true compatibility was involved. The behavior of the F_2 with regard to apomixis should be presented in a future publication.

(Although no factorial explanation of these incompatibilities can be formulated without specific tests, the behavior does not necessarily require an extension of the hypothesis given in [**6**]. If the polyhaploid parent were homozygous for self-incompatibility alleles R_aR_a, all of the offspring, R_aR_b and R_aR_c, would have this allele. They also would have one allele in common with each parent. Under the hypothesis just referred to any plants with one common R allele are incompatible, unless an allele in which they differ is dominant.)

Interspecific hybridization of triploid F_1 plants

Four of the triploid progeny from the cross apomict (42354) × sexual (SP-14) which had originated by fertilization of unreduced eggs—two genomes from the apomictic parent and one from the sexual one (table 3)—were crossed with *P. stramonium*. Their progenies are classified in table 4, *c*. From these four cultures, 58 plants were raised to maturity, and seven interspecific hybrids were found among them. The remaining 51 plants closely resembled their mothers, and pollen measurements of a few of them indicated that they were actually triploids. Thus, their triploid parents were highly apomictic. (No emasculation had been practiced in securing these progenies; the uniformity of the 51 guayule plants, which were the offspring of triploids, indicated that self-fertilization had not taken place; furthermore, no progeny had resulted from 174 achenes when one of the parents was tested for self-incompatibility. Apparently this triploid material is either self-incompatible or progeny arising from selfing have a low survival value.)

Sexuality of artificial tetraploids

In another experiment (**6, 7**) a number of tetraploids had been produced from diploid sexual stock by means of colchicine treatment of seedlings. In order to decide whether doubling of the chromosomes would affect the sexual method of reproduction prevalent in their diploid progenitors, five of these tetraploids were pollinated with *P. stramonium* pollen (table 4, *d*). Of 467 achenes thus obtained, 157 produced seedlings, but only 120 survived; 119 of these were interspecific hybrids, and one plant was pure guayule, with $72\pm$ chromosomes.

Of the 119 hybrids, 82 could be classified by their morphology as typical triploids having two genomes of guayule and one genome from *P. stramonium*. Nine of them, taken at random, had the pollen diameters of triploids. The remaining 37 plants did not look quite typical, but, by the use of pollen diameters, stomate measurements, and trichome morphology, 27 of them were ascertained to be triploids also. Nine had unfortunately died before their level of polyploidy could be determined. Only one hybrid was a tetraploid, and its trichomes indicated that it had equal parts of guayule and *P.*

stramonium; it must have originated by pollination with unreduced *P. stramonium* pollen. Thus, not a single plant that had originated from an unreduced guayule egg cell was found among the 119 hybrids. The one tetraploid pure guayule plant mentioned may have resulted from selfing as no emasculation was practiced, although it could have arisen from unreduced parthenogenesis.

Discussion

The recently published exhaustive monograph on "Apomixis in the Higher Plants" by GUSTAFSSON (**8**) obviates the necessity for any discussion of the literature. Clearly, a factorial analysis of the inheritance of apomixis requires the existence of apomicts and sexuals having the same chromosome count, preferably diploids. The number of known diploid apomictic angiosperms is very small. GUSTAFSSON listed about a dozen, of which the majority exhibit adventitious embryony as the only form of apomixis. Only one—*Potentilla argentea*—of the agamogonous species has been subjected to a genetical analysis, with the conclusion that sexuality dominates (**14**). As GUSTAFSSON pointed out, there is no valid case in the literature where apomixis is dominant over sexuality; where this has been reported, it was due to differences in chromosome numbers. As shown above, guayule is no exception to the rule. Fourteen F_1 plants, sexual $\times$ apomict, and two diploids obtained from the reciprocal cross were sexual. Every one of them had only hybrids in its progeny, and, with one exception, all these hybrids had arisen from reduced ovules. The one exceptional triploid guayule-guayule-*stramonium* might be taken as evidence that a weak tendency to nonreduction is carried over into the F_1. Working with polyploid material, GARDNER (**5**) and POWERS (**15**) also obtained indications of the dominance of the major factors controlling the sexual processes.

In the foregoing were described a diploid (actually trisomic polyhaploid, $2n = 37$), which was apomictic and tetraploids (produced with colchicine) which were sexual—a reversal of what is encountered in wild-type guayule. Thus, it appears that apomixis in this species is not dependent on polyploidy. This viewpoint is strengthened by the occasional occurrence of highly polyploid but sexual material in guayule (**4**). Also, in the authors' experience, certain offspring with more than 100 chromosomes derived from an apomictic tetraploid segregated extensively while in other such high-chromosome plants apomixis prevailed (unpublished). Therefore, it appears fairly certain that the genotype determines the behavior of a given plant and that the chromosome number as such is of little importance. POWERS' (**15**) observation that nonreduction is not influenced by changes in the level of polyploidy but is affected by the genotype lends further support to this opinion. Taken in this light, the behavior of the four F_1 plants with $54\pm$ chromosomes becomes clear. These, as will be recalled, originated from the fertilization of unreduced eggs of the apomictic polyhaploid by pollen of the sexual diploid, and they proved apomictic. Apparently, two doses of the genes for apomixis are dominant over a single dose of sexuality genes, while in the F_1's with equal doses of apomixis and sexuality, sexuality is dominant. The other possibility, that the high chromosome number rather than dominance relationships determines the apomictic behavior of the triploids, appears to be excluded.

This discussion has neglected the complicating fact that apomixis in

guayule is far from being an all-or-none phenomenon in that the proportion of apomictic offspring produced by the various facultatively apomictic strains varies considerably (see [4, 15] for examples). In the present paper it has been shown that the diploid apomictic parent produced a considerably lower proportion of parthenogenetic progeny (table 3) than the triploids (table 4, *c*) which had the same genetic constitution plus one genome from the sexual. This recalls HÅKANSSON's (10) findings in *Poa alpina* that higher chromosome numbers increased the tendency to parthenogenesis. HÅKANSSON (9) stressed the significance of timing upon the fate of the egg cells; polyploidy affects all developmental rates in the organism (18), and it is a distinct possibility that it affects apomixis quantitatively through its influence upon the rates of meiosis, egg-cell division, and pollen-tube growth.

Summary

1. In feral guayule the diploids ($2n = 36$) reproduce sexually, but polyploids are facultative apomicts. A fertile polyhaploid with the diploid number of chromosomes but facultatively apomictic in its reproduction was crossed with a diploid. If the polyhaploid was used as a female, the following progeny classes resulted: maternals, diploid F_1, triploids, and plants with higher chromosome numbers (doubtful cases).

2. The reciprocal cross, sexual diploid × polyhaploid, produced only diploid F_1 plants. Diploid F_1 from both types of crosses were almost entirely sexual; an exceptional unreduced egg was produced in one instance. Production of F_2 and B_1 generations was impaired by incompatibility (not sterility) among F_1 sibs and between them and their recessive parent.

3. Triploids from the cross apomictic polyhaploid × sexual diploid were facultative apomicts.

4. Tetraploids produced by artificially doubling the chromosome number of diploids reproduced sexually on the whole—one exceptional maternal plant in the progeny may have arisen either by selfing or by apomixis.

5. It is concluded that the genes for apomixis in guayule are recessive but that dominance is reversed where two apomictic genomes combine with one sexual one. While apomixis, on the whole, is determined by the genotype, chromosome numbers may have modifying quantitative effects.

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ORIGIN, DEVELOPMENT, AND MORPHOLOGY OF THE INTRAPETIOLAR STIPULES OF ERVATAMIA DIVARICATA[1]

G. C. MITRA

Introduction

The occurrence of intrapetiolar (axillary) stipules is not frequent in dicotyledons, and little work on their origin and nature appears to have been done. Earliest reference to this structure in *Ervatamia* (= *Tabernaemontana*), so far as the writer is aware, was made by GROOM (**4**) in *T. dichotoma*. In giving a general account of bud protection in dicotyledons, he referred to the minute "ledgelike" outgrowths from the inner face of the leaf bases of this plant as stipules.

GOEBEL (**2**) gave an account of the origin and nature of the stipules in *Ficus elastica* and *Artocarpus integrifolia* which he regarded as axillary. He stated that they are formed in the two species by an early union of the two lateral stipules of a leaf by a transverse cushion. My studies (**9**), however, have shown that the stipules in these two species are not axillary.

With a view, therefore, to understanding the real nature of axillary stipules and how their mode of origin and development might differ from that of other types studied by the writer (**5**–**10**), the present investigation was undertaken on *Ervatamia divaricata* (L.) Burkill (= *Tabernaemontana coronaria* Br.).

Material and methods

Terminal vegetative buds of mature plants, in different stages of develop-

[1] Contribution from the Botanical Laboratory, Presidency College, Calcutta, India.

ment, were fixed in FAA solution. Transverse and longitudinal sections cut 8–10 μ thick were stained in various combinations, but ferric chloride–tannic acid–safranin (1) gave the best result. All drawings were made with a camera lucida.

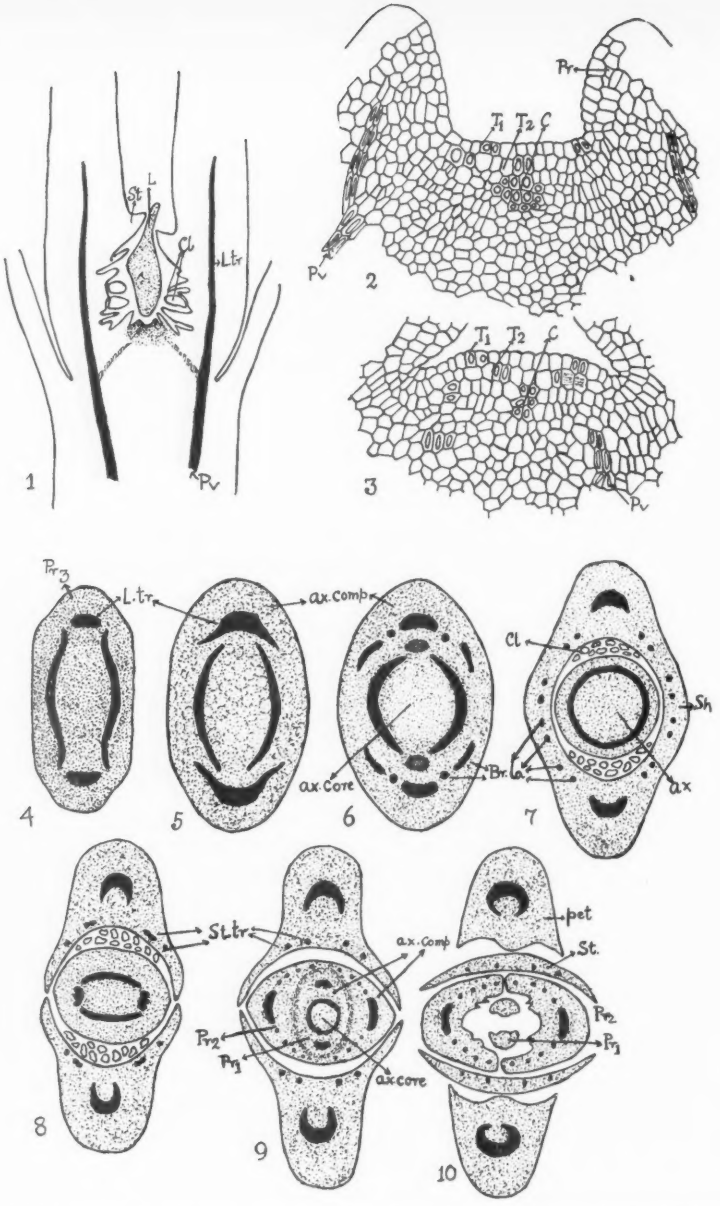
Observations

EXTERNAL MORPHOLOGY.—*E. divaricata*, a small tree which appears to branch dichotomously, belongs to the family Apocynaceae. The stipulate leaves are simple, opposite, shortly petioled, and have entire, wavy margins. Both in transverse (fig. 10) and in longitudinal (fig. 1) sections the axillary position of the intrapetiolar stipule is clearly noticeable. The stipules are "ledgelike" outgrowths on the inner faces of the opposite leaf bases, and colleters are present below them. The leaf bases and stipules form an almost closed chamber in which the shoot apex and the younger leaves are protected (fig. 1).

ORIGIN AND DEVELOPMENT OF LEAF PRIMORDIUM.—The shoot apex is symmetrically convex in outline and consists of two tunica layers and a corpus with no evident zonation (figs. 2, 3). Two opposite foliar primordia are initiated simultaneously. During initiation of a primordium a particular sector of the apex extends transversely, by anticlinal divisions in the tunica and outer corpus, to form the axial component, or foliar foundation ("soubassement foliaires" of GRÉGOIRE [3]), of the primordium. Periclinal and anticlinal divisions in the inner tunica and corpus derivatives composing the axial component bring about the emergence of the primordium in the form of a papillate outgrowth on the side of the apical dome. Provascular cells differentiate acropetally in the axial component and form the median trace of the primordium when it is only 40–50 μ high (fig. 2). Meanwhile the axial components of the opposite primordia extend tangentially along the surface and ultimately meet and fuse about midway around the apex. The embryonic primordium has thus two distinct regions—the axial component which is included in the axis and the relatively undifferentiated free limb. The axial components of the two primordia together form a sheath completely inclosing an axial core (figs. 4–6). The internode is later developed by the united growth of these two regions—mantle and core. The mantle arises from the tunica and corpus derivatives at the flank of the apical meristem and the core from the corpus alone.

After the emergence of the two primordia the apex is flattened or slightly cup-shaped (figs. 1, 2). During the plastochrone rapid anticlinal divisions in the tunica and periclinal and irregular divisions in the corpus, together with an increase in cell volume, result in a shoot apex again convex (fig. 3). This condition is followed by the initiation of the next pair of axial components. Thus, at the beginning and end of a plastochrone the shoot apex presents two distinctive appearances—the concave and convex outlines, respectively. It is, therefore, clear that after the laying-down of a pair of primordia the apex enters into phases of elongation and of surface growth before the next pair can be initiated.

DEVELOPMENT OF LEAF BASE AND ORIGIN OF STIPULES AND THEIR TRACES.—It has been stated that the two axial components form a complete sheath around an axial core. The two grow together for some time before they separate (figs. 5, 6). The node is unilacunar, but, as there is a pair of leaves at each node, two gaps are present (figs. 4, 8). Three bundles make up a leaf trace; in the



FIGS. 1–10.—*Ervatamia divaricata*. Solid black represents vascular bundles, while stippling indicates meristematic nature of cells. Fig. 1, longitudinal section of shoot apex. *St*, stipules; *Cl*, colleters; *L. tr*, leaf trace; *L*, leaf; *Pv*, provascular strand. ×21. Figs. 2, 3, longitudinal sections of apical meristem. ×284. Fig. 2, concave, and fig. 3, convex. T_1, T_2, tunica layers; *C*, corpus; *Pr*, primordium; *Pv*, provascular tissue. Figs. 4–10, serial cross sections of shoot apex. ×30. Fig. 4, 1110 μ below apex; fig. 5, 240 μ above fig. 4; fig. 6, 80 μ above fig. 5; fig. 7, 320 μ above fig. 6; fig. 8, 230 μ above fig. 7; fig. 9, 240 μ above fig. 8; fig. 10, 130 μ above fig. 9. Pr_3, Pr_2, and Pr_1, primordia 3, 2, and 1, respectively, below the apex; *ax. comp*, axial component; *ax. core*, axial core; *ax*, axis; *Sh*, sheath; *St. tr*, stipular trace; *pet*, petiole; *Br. la*, branches of lateral leaf trace.

youngest primordia they can be seen only under high magnification (fig. 11) but are readily visible in the next older primordia.

In still older primordia each lateral trace undergoes branching to form four traces in each wing which arrange themselves along the inner face of the axial component. The sheath then separates from the axis. The free sheath (fig. 7), with sixteen bundles derived from the laterals, continues its upward growth for an average 230 μ and then separates into its component parts (fig. 8). The eight inner strands of each base then unite in pairs so that four bundles, which are arranged along the adaxial face in the central region, remain in each leaf base. The main trace gradually assumes the form of a U by lateral extension and curvature (figs. 8, 9).

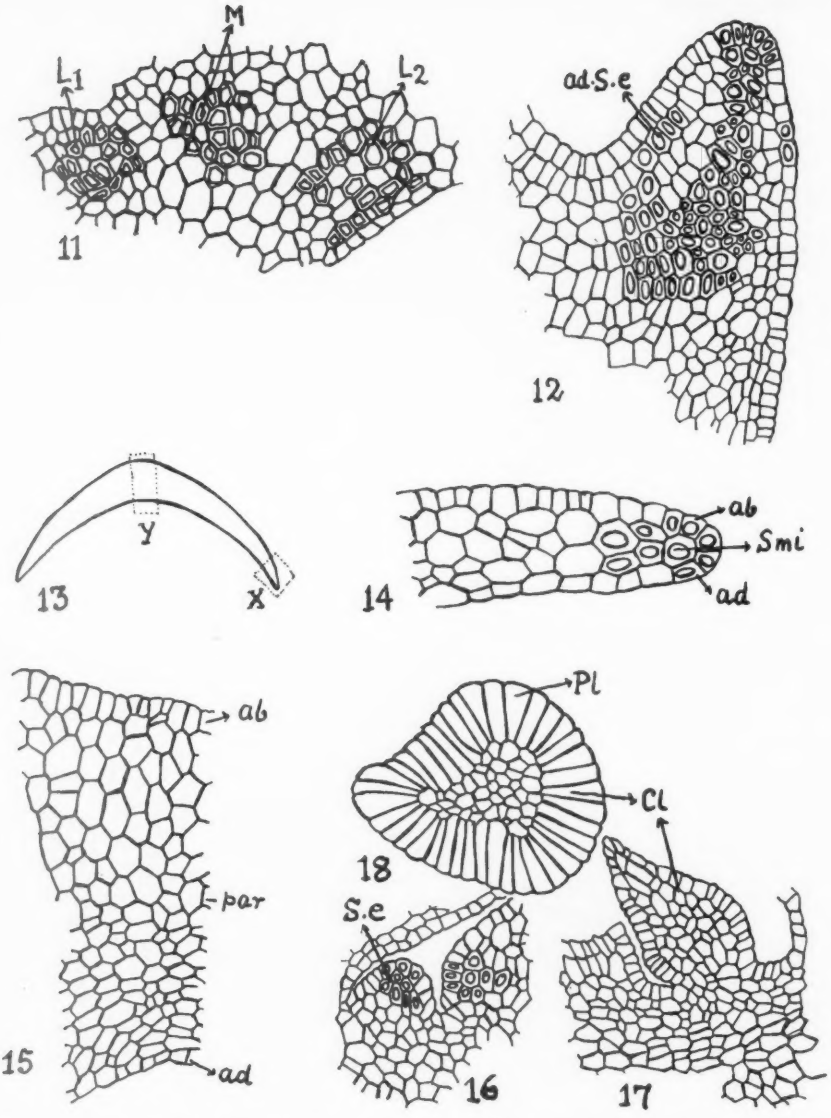
The leaf base grows in length for an average of another 370 μ, and the central region increases in a radial direction. The wings and a narrow strip of the central region containing the four bundles then diverge as a single, undivided piece (fig. 10). This structure, containing four bundles all derived from the lateral strands of the leaf trace, is the rudiment of the intrapetiolar or axillary stipule.

The divergence of the stipule from the central region of the leaf base is initiated by anticlinal divisions of the epidermal and periclinal divisions of the subepidermal layers along the whole inner face. These divisions, plus cell enlargement, bring about a fold on the adaxial surface which, supplied by the branched laterals, develops as the axillary stipule. It is a homogeneous structure and cannot be distinguished into two wings with a transverse cushion connecting them.

The axillary stipule in this case is, therefore, a divergence of the entire inner face of the leaf base. Its traces are derived from the laterals of the three-strand leaf trace.

TISSUE DIFFERENTIATION IN STIPULES.—Vertical growth of the stipule is maintained by a subapical cell in which activity soon ceases. A marginal meristem is continuous around the edge and by anticlinal divisions forms the adaxial and abaxial epidermis. Cells of a submarginal meristem divide periclinally, and their derivatives, by both anticlinal and periclinal divisions, form the other layers of the stipule (figs. 13, 14). Owing to irregular divisions of the derivatives of the submarginal cells, the subepidermal layer differentiated from the latter cannot be distinguished from the other layers of the stipule. The mesophyll of the stipule is not differentiated (fig. 15). Early maturity, small size, and thick walls of the cells are apparently correlated with their short-lived character and protective function.

DEVELOPMENT OF COLLETERS.—Colleters are developed from the inner face of the leaf base below the stipule. Adaxial epidermal cells divide periclinally, initiating papillate outgrowths (fig. 16) which by further divisions of both epidermal and subepidermal cells give rise to the colleters (fig. 17). The tissue of the colleters is differentiated into a peripheral layer of palisade-like cells, anticlinal derivatives of epidermis, and a central core of thin-walled cells, periclinal and irregular derivatives of subepidermal cells (fig. 18). They are glandular, hairy coverings containing mucilage and resinous substances. No laticiferous tubes or vascular tissues were found in the colleters, and the cells showed no sphaerocrystals or raphides which have been reported by GROOM to occur in *T. dichotoma* (4).



FIGS. 11–18.—*Ervatamia divaricata*. Fig. 11, cross section through node showing three bundles of leaf trace; median (*M*) and laterals (L_1, L_2). ×476. Fig. 12, longitudinal section through stipule in adaxial subepidermal layer (*ad.S.e*) and its derivatives. ×286. Figs. 13–15, cross sections of stipule. Fig. 13, diagram. ×20. Fig. 14, detail of *X* in fig. 13 showing marginal growth of stipule. ×295. Fig. 15, detail of *Y* in fig. 13 showing nature and differentiation of mesophyll cells. ×223. *Smi*, submarginal initial; *ad*, adaxial; *ab*, abaxial; *par*, parenchymatous cells. Figs. 16–18, colleters. ×267. Fig. 16, longitudinal section showing initiation in subepidermal layer (*S. e*). Fig. 17, longitudinal section of mature. Fig. 18, cross section showing palisade-like peripheral cells (*Pl*).

Discussion

The fact that the stipular tissue originates as a single homogeneous piece does not support, for this plant, GOEBEL's (2) contention that a new formation connects a pair of lateral stipules to form the axillary stipule. The upgrowth of the adaxial surface of both the central region and the wings of the leaf base during the formation of a single stipule is continuous, and the middle region cannot be differentiated from the lateral portions at any stage of its development. The constant association of a stipule with branches of the lateral strands of a leaf trace, as observed by SINNOTT and BAILEY (11) in a large number of species, and the acropetal differentiation of the stipular strand in *E. divaricata* and in other species studied by the writer (5–10), suggests that the upgrowth of the adaxial surface as a ledgelike outgrowth may be influenced by the branches of the laterals activating anticlinal and periclinal divisions of the epidermal and subepidermal layers which first form a surface fold and then, by subsequent divisions, increase in length.

The position and mode of development show that the stipules are intrapetiolar, or axillary, in the sense that they grow out from the inner faces of the leaf base and occupy an axillary position.

The origin and formation of the stipules in *E. divaricata* differ from other kinds of stipules in that both the arms and the adaxial portion of the central region of the leaf base take part. Their vascular supply corresponds to other types of stipules—from the laterals of the leaf trace.

Summary

1. The nature and development of the intrapetiolar stipule of *Ervatamia divaricata* (L.) Burkill has been described.

2. The embryonic leaf primordium has two distinct regions—the axial component which is included in the axis and the relatively undifferentiated free limb. The two opposite axial components together form a sheath completely inclosing an axial core. The sheath ultimately separates from the axis and then into its components. The wings and a narrow strip of the central region of each leaf base containing the stipular strands then diverge as a single undivided piece which develops into the intrapetiolar stipule. The upgrowth of the stipule from the inner face of the leaf base is initiated by divisions of the epidermal and subepidermal layers of the adaxial surface.

3. Provascular cells differentiate acropetally in the axial component. Three bundles make up a leaf trace. The laterals branch in the axial component, and the branches ultimately form the stipular strands.

4. Colleters are developed from the inner face of the leaf base below the stipule. No vascular tissue or laticiferous tubes are present in them.

5. The mode of origin and nature of the intrapetiolar or axillary stipule differ from the other types of stipules studied by the writer and does not support, for this plant, GOEBEL's interpretation of axillary stipules.

The author expresses his gratitude to Prof. G. P. MAJUMDAR, under whose guidance the work has been carried out.

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MALEIC HYDRAZIDE AS A PLANT GROWTH INHIBITOR[1]

AUBREY W. NAYLOR AND EDWIN A. DAVIS

Introduction

Many substances have been synthesized during the last two decades which cause profound alterations in morphological expression. Most of these are known as hormones or hormone-like substances. Maleic hydrazide, one of the latest to be described, has been found to have striking effects on plant growth, development (**9, 11**), and flowering (**8**). But the question may well be raised whether maleic hydrazide should be considered a hormone-like substance or a growth inhibitor.

Most of the hormone-like substances such as indoleacetic acid, naphthaleneacetic acid, indolebutyric acid, beta-naphthoxyacetic acid, 2,4-dichlorophenoxyacetic acid, and 2,3,5-trichlorophenoxyacetic acid are associated with atypical growth which may take the form of excessive root initiation and development, growth of fruits without pollination, or fasciation of stems and roots. Although the presence of these growth substances may result in inhibition in growth of one tissue or organ, such inhibition is usually accompanied by rejuvenation and activation of tissues in some other part of the plant (**14**). Immediate changes in metabolism, including redistribution of food (**7**), protein synthesis (**4**), and respiration (**1, 6**), are also known to accompany the presence of such substances. Although at certain concentrations in certain plants these compounds may act as metabolic poisons, they cannot be considered general growth inhibitors.

The true growth inhibitors do not ordinarily promote growth at any concentration. Agents antagonistic to metabolites often act through interference in utilization of certain vitamins or amino acids or intermediate products in the respiratory cycle. Single examples will

[1] This investigation has been aided in part by a grant from the Rockefeller Foundation.

suffice. Para-aminobenzoic acid, one of the B vitamins, and sulfanilamide act competitively in the growth of bacteria and roots (**2, 13, 16**); canavanine inhibits the utilization of L-arginine as shown in certain strains of *Neurospora* (**5**); and oxidation of succinate can be prevented by addition of malonate (**10**) to the substrate, thereby closing an important path in respiration.

It has been our aim to characterize some of the gross responses to maleic hydrazide of a number of economically important plants in several families in terms of bud growth, stem growth, root growth, and flower development, the ultimate objective being to determine whether maleic hydrazide is a "selective" growth suppressant or a "general" suppressant, i.e., whether it is a hormone-like compound or a growth inhibitor.

Material and methods

In a few instances, particularly when effects of maleic hydrazide on flower development were being determined, fairly mature plants were employed. For the most part, however, where gross morphological response was being studied, seedlings were used. Oats (var. Victory), wheat, redtop grass, hybrid maize (Wisconsin #525), pea (var. Perfection), peanut, sunflower, *Xanthium*, tomato, tobacco, and cotton were treated under similar conditions and with similar concentrations. Seed was planted in $4\frac{1}{2}$-inch pots filled with a well-mixed and fertilized garden loam. To pots seeded with grasses a surface layer of vermiculite was added to aid in reducing loss from damping-off fungi. This procedure proved quite successful, and uniform stands were readily obtained. Redtop grass was seeded to flats at the rate of 2.5 gm. per sq. ft. These flats were subsequently treated as small plots.

Maleic hydrazide was supplied by the manufacturer as a 30% solution in diethanolamine. One experiment was performed using the diethanolamine salt, but all the others were performed with aqueous solutions of crystalline maleic hydrazide. Repeated crystallization gave a clean, well-defined product with a sharp melting point. Maleic hydrazide is not very soluble in water, but a 0.50% solution can be kept at pH 6.0 at 5° C. indefinitely, without crystal formation. At pH 4.5, however, crystals do form at this temperature. Although solutions were always freshly prepared before use, it was found that at pH 4.0 they could be kept without apparent deterioration at room temperature. They were always adjusted to pH 6.0 before application to the plants.

A small medicinal-type atomizer was employed in all experiments where leaves were treated. The reservoir was graduated for ease in determining the amount of substance used. Leaves and stems were always sprayed in such a manner that they had a film of the desired solution on all surfaces. The concentrations used were almost always 0.00, 0.05, 0.1, 0.2, and 0.4%. In all cases of spray application a spreading agent called Emulphor EL or Aerosol OT was employed at the rate of 1 drop per 100 ml. of solution. In none of the experiments was there evidence of these spreading agents influencing growth in any way.

Following treatment the plants were randomized in a Latin square on a greenhouse bench and so remained until the experiment was terminated. This arrangement provided five replications of each treatment and was used in all cases except where so designated.

Experimentation

Reaction of grasses—redtop, wheat, maize, and oats

Schoene and Hoffmann (11) suggested the possibility of using maleic hydrazide in suppressing growth of lawn grasses. Since few substances have proved effective in suppressing growth of grasses, we ran tests on four—one lawn grass and three cereals—to determine if maleic hydrazide is differentially effective.

Greenhouse flat-sized plots of seedling redtop grass were sprayed at the rate of 7.5 ml. per sq. ft. with concentrations of maleic hydrazide ranging from 0 to 0.4% when the plants were 4.5 cm. tall. Within 5 days there was a noticeable difference in height in the different groups. The plants treated with 0.2 or 0.4% solutions had withered leaves, whereas those treated with 0.1 or 0.05% solutions were materially inhibited in growth but did not show much damage to the leaves. A few days later, however, it was clear that seedling redtop cannot be safely treated with maleic hydrazide because of the great danger of toxicity.

Wheat grown densely and sprayed with maleic hydrazide at the rate of 20 ml. of solution per pot in the usual concentration series showed differences in growth, in contrast with the controls, within 7 days after treatment. Twenty days after spraying there was relatively little difference in height among the different treated groups (fig. 1*B*). The treated plants were then greener in appearance than the controls. New leaves, however, were not produced by the treated plants, whereas those not treated did show new terminal growth. Three days later axillary branches were being produced on the plants treated with the 0.05% solution.

When the wheat was harvested, 23 days after treatment, casual examination indicated that root growth had not been so severely affected by the treatments with maleic hydrazide as had that of the tops. Fresh-weight figures bore this out, and dry-weight figures were equally clear (table 1). Although dry matter in the tops of treated plants was only half as great as in the controls, there was no appreciable difference in the amount of dry matter in the roots.

Victory oats treated 9 days after planting reacted to maleic hydrazide in a manner similar to wheat. Top growth was suppressed an equivalent amount by all concentrations used (fig. 1*D*). At harvest dry weight of the tops of treated plants was only 60% as great as the controls, while root growth was approximately 69% of the controls (table 1). It thus appears that, although root growth was suppressed, top growth was inhibited slightly more, thereby causing the top-root ratio to be smaller than in the controls.

The largest grass used in the experiments was maize (Wisconsin #525). Two different stages in growth were selected for treatment—seedling and microsporogenesis stages. For the seedling studies the seed was planted quite densely in the first experiment and singly in the second. Where it was desired that the plants grow to maturity, they were grown singly in 2-gallon crocks. This amount of soil allowed excellent growth.

Seedling maize was treated with 0.0, 0.05, 0.1, 0.2, or 0.4% maleic hydrazide 8 days after planting the seed. Within 4 days there were visible differences in amount of leaf growth between treated plants and controls. About 8 days after spraying, viscous droplets appeared on the abaxial surface of the leaves (fig. 2*D*). Since these appeared at random over the

FIG. 1.—General effects of single-spray treatment of maleic hydrazide on growth of representative plants. *Left to right: A, B, C, D, E* — control, 0.05, 0.1, 0.2, and 0.4%. *A*, peas (Perfection), 17 days after being sprayed. *B*, wheat, 20 days after treatment. *C*, peanuts, 14 days after treatment. *D*, oats (Victory), 27 days after treatment. *E*, sunflower, 18 days after treatment. *F*, tobacco (Turkish), 32 days after treatment: *left to right*, control, 0.05, 0.1, 0.2, 0.4, and 0.8%. Note in tobacco no flowers were produced at concentrations above 0.1%, and no axillary buds developed at concentrations of 0.4 or 0.8%.

whole surface, it is probable that they came from the intercellular spaces through the stomates. Because they were sweet to taste, we were led to speculate that maleic hydrazide in some way altered enzymatic activity, resulting in uniformly affected by the concentration range employed.

The maize seedlings were harvested 20 days after treatment; roots were carefully washed free of debris, tops were separated from the roots, and both were

TABLE 1

EFFECT OF SINGLE TREATMENT WITH MALEIC HYDRAZIDE UPON DRY WEIGHT (GM.) OF ROOTS AND SHOOTS OF WHEAT, OATS, AND MAIZE

Maleic hydrazide conc., %	No. of plants	Dry wt. total tops	Dry wt. total roots	Av. wt. tops	Av. wt. roots	T/R
	Wheat					
0.00	470	16.41	3.40	0.035	0.0072	4.86
.05	629	11.80	3.27	.019	.0068	2.79
.10	591	11.70	3.97	.020	.0067	2.98
.20	651	11.01	3.96	.017	.0061	2.79
0.40	565	9.95	4.00	0.018	0.0071	2.54
	Oats					
0.00	384	15.32	3.40	0.040	0.0088	4.54
.05	472	11.28	2.96	.024	.0063	3.81
.10	509	11.33	3.03	.022	.0060	3.67
.20	391	9.28	2.41	.024	.0062	3.87
0.40	404	9.93	2.40	0.024	0.0059	4.07
	Maize					
0.00	72	21.59	10.85	0.295	0.151	1.95
.05	77	15.28	5.78	.198	.075	2.64
.10	75	16.20	5.94	.216	.079	2.73
.20	72	15.63	5.62	.217	.078	2.78
0.40	74	14.55	6.05	0.196	0.082	2.39

sugar accumulation. Studies on sugar metabolism as affected by this substance were instituted and are being reported elsewhere. Shortly after the sugar exudate appeared, anthocyanin pigmentation became especially marked in plants which had received the highest concentration of maleic hydrazide. A few days later all treated plants showed approximately equal amounts of anthocyanin pigmentation. Height of plants, too, was weighed. After thorough drying, weight was again determined. Both top and root growth were suppressed by all concentrations of maleic hydrazide used (table 1). Root growth of maize, unlike that of wheat and oats, was inhibited more than that of shoots. Thus, although the tops of the treated plants had produced 60% as much growth as the controls at the time of harvest, the root systems had grown only 50% as much.

FIG. 2.—*A*, effect of 0.025% maleic hydrazide treatment at about time of microsporogenesis upon ear development in maize. Note good development of silks. *B*, effect of 0.05% solution on ear and tassel development; tassel did not emerge from sheaths, and no silks were produced on ears. *C*, Turkish tobacco plants: *left*, control with numerous mature fruits; *right*, plant the same age which received 100 ml. of 0.4% maleic hydrazide solution added to soil about 7 days before flower buds became macroscopic; flowers did not develop and leaves continued to expand. *D*, blade of seedling maize plant showing droplets of sugar solution which appeared over blade surface 8 days after treatment with 0.1% maleic hydrazide.

In a repetition of the experiment just described, the sequence of visible changes after treatment were as before: slowing down in growth, loss of chlorophyll in spots on the lower part of the youngest leaves, appearance of droplets containing sugar, and red to purple anthocyanin pigmentation. All the plants treated with 0.1% maleic hydrazide or higher concentrations eventually died. All the plants treated with 0.05% solutions lived as long as the controls, but their growth pattern was atypical. Soon after treatment the plants ceased growing in height. At least one axillary bud per plant then began active growth, which is most unusual for maize. The new leaves were wrinkled or curly, and there was no tendency for the new growth to be upright; in fact, it was very much like the growth of "lazy" corn. After the branches had grown about $1\frac{1}{2}$–2 feet, they began to show signs of negative geotropic response and slowly assumed an upright position. These plants finally flowered, producing staminate inflorescences with some pistillate flowers on the central spike.

In a third experiment with maize, older plants were used. These were about 75 cm. tall and, judging from the position of the tassel within the leaf sheaths, were at the microsporogenesis stage when sprayed. The individual plants were treated at the rate of 20 ml. per plant with 0.0, 0.025, 0.05, 0.1, or 0.2% maleic hydrazide solutions adjusted to pH 6.0.

These plants showed evidence of dosage response. Those receiving the highest concentration were inhibited most, whereas those treated with the lowest concentration showed relatively little inhibition. The 0.2% concentration caused almost immediate cessation in growth. Finally these plants died from the tip back after producing a considerable quantity of anthocyanin and secreting droplets of sugar. Tassel development also failed to occur in those plants treated with the 0.05% solution, but two ears per plant did develop (fig. 2*B*). No silks, however, were produced. Sugar droplets were secreted, and anthocyanin was produced. Clearly the plants treated with 0.025% solutions were least affected by maleic hydrazide so far as vegetative development was concerned; nevertheless, these plants had narrow leaves and smaller stems but were just as tall as the controls. Tassels were produced, but no pollen was formed. Pistillate flowers, on the other hand, were produced on these stalks (fig. 2*A*) and were fertile. The apparent selective toxicity to flower fertility may be caused by differences in time of sporogenesis, but further evidence is necessary to establish this point.

Reaction of legumes

Two legumes—pea and peanut—were tested. Seedling peas (var. Perfection), 10 days old, were sprayed and arranged in a Latin square in a glass compartment in the greenhouse which was maintained at 70° F. Every concentration of maleic hydrazide used suppressed growth equally (fig. 1*A*).

Peanuts also proved sensitive to maleic hydrazide. Differences in height were distinguishable within 5 days after spraying. Two weeks later the plants appeared as shown in figure 1*C*. Flowers began to appear on the untreated plants 6 weeks after planting. All these plants flowered abundantly. Those plants which had been treated with maleic hydrazide, however, even with the lowest concentration, flowered very poorly. Those that had received the 0.05% spray averaged less than one flower per plant; those receiving higher concentrations had no flowers.

All concentrations used caused loss in apical dominance in peanuts. Axillary branches developed fairly quickly on the plants sprayed with 0.05% maleic hydrazide and appeared normal. Much later the plants treated with the 0.1% solution also produced axillary growth. The new leaves on these were very narrow and chlorotic and gave evidence of the interveinal tissue having grown more rapidly than the veins.

When the peanut plants were harvested—at the age of 6 months—the controls were still vigorous and growing well and were still taller than any of those which had been sprayed with maleic hydrazide. Axillary growth of the plants treated with the 0.05 or 0.1% solution was good. All those receiving the 0.2% treatment were alive but were only one-third to one-half as tall as the controls; and all those treated with the 0.4% solution were dead.

Reaction of compositae

Two composites—sunflower and *Xanthium*—have served as test plants in these experiments.

Treated sunflower plants attained good size though not equal to that of the controls. Increase in height was most markedly inhibited by the 0.2% and 0.4% treatments. Of all the plants tested, sunflower appeared to have water relations most seriously interfered with by maleic hydrazide. The lower leaves remained flaccid for some time in all treatments, and with the two highest concentrations the stem tips bent over and grew horizontally (fig. 1*E*).

Those sunflowers sprayed with 0.05, 0.1, or 0.2% solutions had interveinal chlorosis on the lower leaves and produced axillary shoots, which is a rare occurrence for sunflower under normal conditions. With concentrations of 0.1% or above, neither flowers nor flower buds appeared during the course of the experiment. One of the responses not noted in any of the other plants was the presence of very much enlarged petioles on the leaves of plants sprayed with 0.2% maleic hydrazide. Hypertrophy or hyperplasia—which was not determined—appeared to be greatest at bases of the petioles.

Xanthium reacted quickly to treatment. All concentrations from 0.05 to 0.4% were associated with loss in apical dominance. Terminal growth apparently stopped immediately after treatment. Axillary bud development was also suppressed in all except the plants treated with the lowest concentration. Although increase in number of cells at the apex was stopped and elongation of internodes was prevented (figs. 3*A*, *B*), increase in diameter of the stems continued to occur, thereby resulting in a stem of as great diameter and in many cases larger than the controls. The stem tip thus appeared quite obtuse. As noted previously for several other species, 0.05% maleic hydrazide suppressed growth of the apical meristem, but axillary growth seemed stimulated. In *Xanthium* every axillary bud on these plants, including the cotyledonary buds, began to grow at about the same time (fig. 3*A*). The new leaves were narrow, sometimes straplike, chlorotic in appearance, and, because of unequal growth of the veinal and interveinal tissue, quite rough in texture.

In an effort to determine the effect of low concentrations of maleic hydrazide, when intermittently supplied, on growth characteristics of *Xanthium*, six concentrations—0.00, 0.01, 0.02, 0.04, and 0.05%—were employed. The plants were so arranged that a single blade was in the appropriate solution during the day

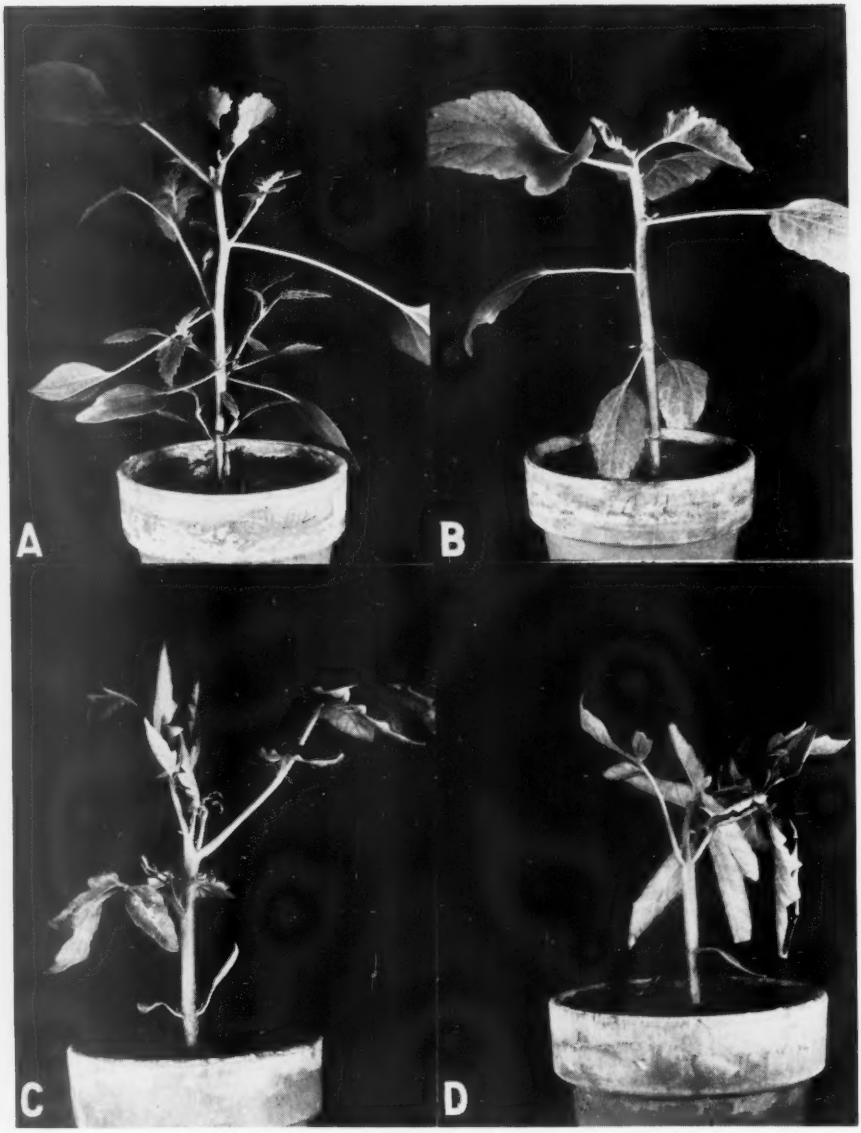

FIG. 3.—Response of *Xanthium* (*A*, *B*) and tomato (*C*, *D*) to 0.05 (*A*, *C*) and 0.1% (*B*, *D*) maleic hydrazide. Note loss in apical dominance in both species at both concentrations. At lower concentration auxillary buds are developing at each node including cotyledonary node. Leaves on these shoots, however, are not normal. At higher concentration axillary buds as well as terminals were inhibited. Increase in stem diameter was not inhibited, though increase in length was.

and in the air during the night. After 7 days this treatment was discontinued. Within 2 weeks typical symptoms of maleic hydrazide poisoning were apparent in the new growth. Thus, it is clear that this substance can be absorbed, translocated, and exert its effects at a distance from the point of treatment. When the plants were photoinduced to flower, inflorescences appeared after considerable vegetative growth. These showed marked phyllody.

Response of solanaceae

The relative sensitivity of one variety of tomato to maleic hydrazide was described by Schoene and Hoffmann (11). Their observations have been confirmed and extended with the Rutgers variety. Young plants, 21 days old, were sprayed at the rate of 5 ml. per plant. The concentration series ranged from 0.05 to 0.4%.

All the tomato plants receiving maleic hydrazide were stunted by the treatment. Those sprayed with the 0.05% solution, however, grew more than any except the controls. Plants receiving all other concentrations were remarkably similar in height for some time after treatment. Those treated with 0.4% maleic hydrazide died as a result of treatment. In all except the controls there was a tendency for the leaflets to roll inward from the edges. This was most pronounced in plants treated with the highest concentrations. There was also a strong tendency toward production of anthocyanin pigmentation along the veins, particularly on the abaxial surface. The presence of maleic hydrazide also seemed to result in abnormally large stem diameters. Those of plants sprayed with 0.05% solutions were characteristically thick from the tip to the soil line (fig. 3*C*); while those treated with higher concentrations were characteristically wider in diameter in the first internode than from ground level to the first node (fig. 3*D*).

Shortly after treatment the axillary buds of the young tomatoes began to grow; cotyledonary buds also developed. Although the first new growth was quite malformed, normal leaves were eventually produced on these shoots.

Tobacco, the other solanaceous plant used, was treated both with sprays and with additions to the soil. The first tests were run with the diethanolamine salt; the other two, with maleic hydrazide. Unlike all the other plants, tobacco was tested only at the mature stage at or very near the point of floral initiation.

Six concentrations (0.0, 0.05, 0.1, 0.2, 0.4, and 0.8%) of the diethanolamine salt were used in the test on Turkish tobacco plants 1-meter tall and just initiating flower buds. Response as measured by growth in height was somewhat different for each concentration. The two highest concentrations stopped all elongation, killed the flower buds, and prevented axillary bud development. The intermediate concentrations (0.1 and 0.2%) prevented flowering but did not inhibit axillary bud development (fig. 1*F*). Leaf size was affected by the treatment; in contrast with the controls the topmost leaves became as enlarged as the lower ones.

The new axillary and terminal growth in the plants treated with 0.1% maleic hydrazide was interesting because of the similarity to "frenching," a disease of tobacco which has been attributed to many causes. The leaves, whether terminal or on axillary branches, were narrow and strap-shaped; in some cases they were quite stringy. There was also some roughness and curl because of unco-ordinated growth in veins and inter-

veinal tissue and also a limited chlorosis.

In another experiment with Turkish tobacco maleic hydrazide solution was added to the soil rather than as a spray on the leaves. The plants were approximately 54 inches tall, about 3 months old, in excellent condition, and, according to previous experience, should have flowered within a short time. Solutions of the substance were prepared in the concentrations 0.00, 0.05, 0.1, 0.2, and 0.4% and adjusted to pH 6.0. After allowing the soil to dry out somewhat, each plant was watered with 100 ml. of the appropriate solution. Within 25 days the controls had all flowered, but 2 months after treatment, at harvest, none of the treated plants had flowered. There was no new growth—terminal or axillary—and flowering was completely suppressed in the plants receiving the highest (0.4%) concentration (fig. 2*C*). Some of the plants receiving lower concentrations did show axillary branching and decided evidence of "frenching," as shown when plants were sprayed.

We also wished to see if maleic hydrazide would kill the flowers if the flower buds alone were treated after reaching macroscopic size. Concentrations up to 0.4% were used. None of the concentrations proved effective, and all buds produced mature flowers.

Reaction of Malvaceae—cotton

The only member of the Malvaceae we have yet tested is cotton—variety Deltapine 14. When the plants were 7 weeks old, the usual spray procedure with five replications and concentrations of maleic hydrazide up to 0.4% was followed. The effects of treatment were readily measurable within 10 days after spraying and were very marked within 3 weeks. One month after treatment those plants sprayed with the 0.05% solution were about as tall as the controls. But all concentrations above 0.05% did cause inhibition in growth. The greatest inhibition occurred at the highest concentration, where little or no elongation took place after treatment (fig. 4*A*).

Growth of cotton leaves following spraying was similar to that of other broad-leaved plants. Leaves which were already unfolded expanded to a larger surface area and became darker green in color than leaves of controls. But leaves which were primordial at the time of treatment developed in a malformed manner. They were not normal in size, shape, or development of tissues (fig. 4*B*). As in other treated plants anthocyanin developed excessively along the petioles.

Discussion

The response to maleic hydrazide of all eleven species tested is quite different from their response to any other known substance. This is true in degree and kind. Results thus far obtained show that reaction to maleic hydrazide is remarkably uniform from species to species. Within the concentration range used the effects were strikingly similar for plants as distantly related as grasses, legumes, composites, and nightshades. With hormone-like compounds such uniformity in response is not encountered.

Treatment with concentrations as low as 0.05% maleic hydrazide was associated with loss in apical dominance in almost every species. Since the terminal bud certainly was not killed by the lower concentrations, it would appear that this substance in some way interferes with the regulatory mechanism whereby the stem tip ordinarily keeps the axillary buds in check. Maleic hydrazide should, therefore, prove a useful tool in the study of phenomena associated with apical dominance.

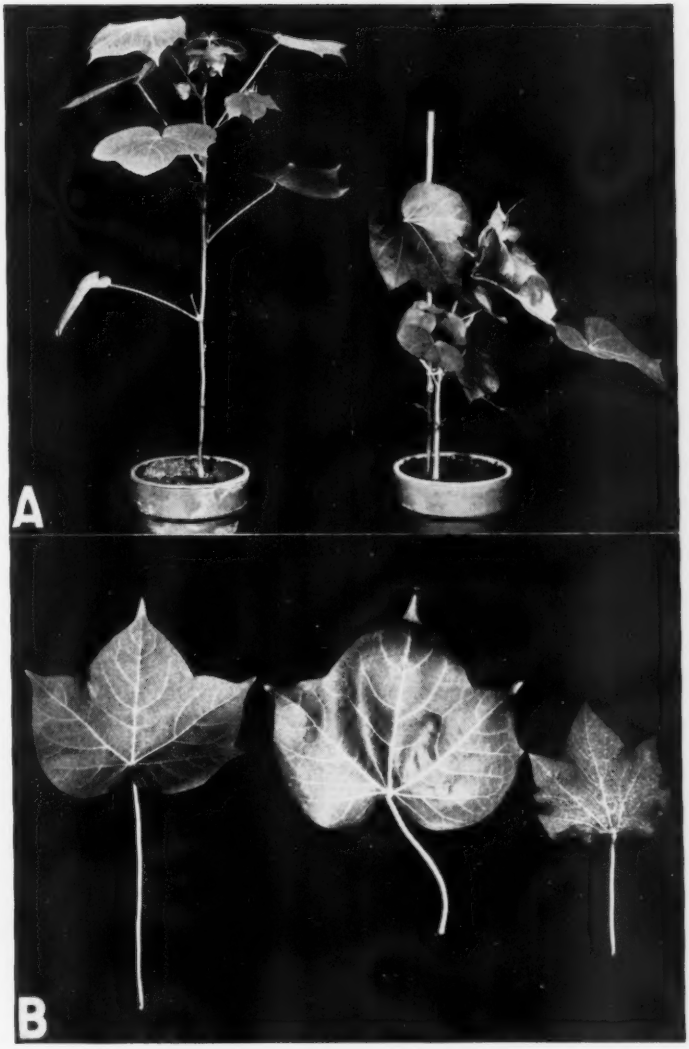

FIG. 4.—Reaction of cotton (Deltapine 14) to maleic hydrazide. *A*, height and leaf size of control (*left*) and of plant treated with 0.4% maleic hydrazide (*right*) 26 days after spraying. *B*, leaves of control and of plant sprayed with 0.05% maleic hydrazide: *left*, from control; *center*, from comparable position on treated plant; *right*, leaf produced subsequent to treatment.

Behavior of seedlings and of older plants of the same species when treated once with maleic hydrazide is not quite the same. Clearly there is some loss in sensitivity with age, though not very great. Thus seedlings are more likely than older plants to be killed by a given concentration of the substance. Also in the older plants there appears to be, when measured on a growth-in-length basis, a more direct relationship between growth and concentration of maleic hydrazide than is found with seedlings.

Unlike ordinary hormone-like substances, maleic hydrazide in no case induced the initiation of root primordia or caused fasciation or true epinasty. The effects on vegetative growth were in every species first a cessation of activity of the terminal meristem; second, a cessation in elongation of the internodal regions; third, an increase in diameter of the stem. The new leaves produced by axillary buds, which grow only on the plants treated at the lower concentrations, were always abnormal in appearance. The meristems of these branches were not permanently affected, however, and eventually normal leaves were produced. Since none of the observed phenomena can be considered to represent stimulation to growth, it seems that maleic hydrazide should be, at least for the present, considered a growth inhibitor.

Because of the uniform effectiveness of maleic hydrazide as a growth inhibitor it seems unlikely that this substance will be generally useful as a selective herbicide. CURRIER and CRAFTS (3) suggested that maleic hydrazide might be used as a weed-killer in cotton fields. This suggestion, however, must be accepted with some caution. The cotton variety used in our tests proved virtually as sensitive to maleic hydrazide as other crop plants. It would appear, therefore, that use of the substance in weed control in field production would be limited to periods after almost all growth of the crop plant has ceased. And even then there is the possibility that the developing embryos might be affected. This point, however, still remains to be determined.

Which mechanism or mechanisms maleic hydrazide inhibits is a matter of conjecture. It is hoped that more light will be thrown on this question in the near future. Several lines of investigation are suggested as a result of these experiments. One of them which holds promise arose from the observation that some of the early changes in appearance accompanying treatment with maleic hydrazide resemble symptoms associated with mineral deficiency. The development of a dark-green color, followed by the appearance of anthocyanin, then by some chlorosis, remind one of phosphorus deficiency. The possibility therefore presents itself that maleic hydrazide interferes in some way with phosphorus metabolism. This lead is currently being thoroughly exploited.

Some of the responses noted also suggest the possibility that maleic hydrazide is an antagonist of indoleacetic acid. Loss in apical dominance as manifested by rapid development of axillary buds of all species tested and the excessive enlargement of leaves of tobacco, *Xanthium*, and cotton were in many ways the physiological equivalent of changes following decapitation. Another indication of interference in auxin metabolism was the "lazy" maize condition which occurred in the axillary growth of maize. Such inability to stand erect in an inbred strain of maize which is structurally quite capable of supporting itself but is nevertheless semiprostrate in habit has been attributed by VAN OVERBEEK (15) and SHAFER (12) to deficiencies in auxin metabolism. Careful investigations on

the action of maleic hydrazide upon auxin content are therefore indicated.

Summary

1. General effects of maleic hydrazide upon eleven species belonging to five distantly related families of plants have been determined.

2. Data thus far assembled indicate that maleic hydrazide has remarkably similar effects on monocotyledonous and dicotyledonous plants. This generalization holds in terms of morphological response of plants of a given physiological age group to a given concentration of the substance.

3. Seedlings are most sensitive to maleic hydrazide; but it can inhibit growth at any stage up to maturity without killing the plant. Generally, in the plants tested, a spray of 0.4% concentration will cause all meristematic growth to cease.

4. Evidence was obtained which indicates that maleic hydrazide may readily enter the plant through either the leaves or the roots and be rapidly transported to regions of meristematic activity.

5. The usual sequence of symptoms produced in treated plants is: (*a*) loss of apical dominance; (*b*) expansion of leaves already formed, often to a size greater than the controls; (*c*) production of a noticeably darker green color than the controls; (*d*) increase in production of anthocyanin pigmentation; and (*e*) some chlorosis. Subsequent behavior depends to a large extent upon the amount of maleic hydrazide given and to some extent upon the age of the plant and upon the species.

6. At the lowest concentrations, generally 0.05%, axillary buds of seedlings begin to grow very soon after treatment. Virtually all axillary buds grow at the same rate on treated plants though the cotyledonary buds develop a little after the others. Usually the leaves on the new branches are malformed and may be quite "stringy"; often the veins grow more slowly than the interveinal tissue. This results in a rather rough texture. In some of the plants which were allowed to grow a very long time after treatment, normal-appearing leaves were finally produced.

7. At the higher concentrations of maleic hydrazide (0.2–0.4%) vegetative bud development is slow to occur and may not occur at all.

8. Root as well as top growth is affected by maleic hydrazide, but under the conditions of these experiments roots were less affected than the tops. Wheat root growth was not affected by the concentrations used; root growth in oats was suppressed almost as much as that of the tops; but maize roots were suppressed more than the tops.

9. Flowering in the plants tested was remarkably suppressed by maleic hydrazide. Sunflower and peanut treated in the seedling stage rarely produced any flowers. At a concentration of 0.025% maleic hydrazide caused sterilization of the staminate inflorescences in maize when the plants were treated at about the time of microsporogenesis; but fertile pistillate flowers were produced under similar conditions. Flowering in Turkish tobacco was prevented either by adding maleic hydrazide to the soil or by spraying the foliage. In photoperiodically induced *Xanthium* phyllody commonly occurred after treatment.

10. The argument that maleic hydrazide may have considerable theoretical as well as practical value as a growth inhibitor is developed.

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SOME METHODS USED IN TRACING RADIOACTIVE GROWTH-REGULATING SUBSTANCES IN PLANTS

JOHN W. MITCHELL[1] AND PAUL J. LINDER[2]

REMOTE CONTROL APPLICATOR

In treating plants with isotopically tagged growth-regulators, it is often necessary to apply a measured volume of the solution containing the tagged compound to a known surface area on the plant. The volume required in some experiments is relatively small, for example, 0.01–0.02 ml. Also, it is often desirable to limit the size of the treated area so that the treatments will not spread and thus can be standardized. A method of limiting the treatment to the desired surface area by means of a lanolin ring has been described previously (1).

An applicator was made to hold a pipette at a distance of approximately 2 feet from the operator. An aluminum rod approximately 65 cm. long and 5 mm. in diameter was supported by means of two 13-mm. aluminum angles (*A* and *B*, respectively, in fig. 1). One end of the rod was threaded and screwed through a plate into a metal box, *C*, where it rested against a movable plate, *D*. By turning the rod, pressure could be exerted so as to

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press plate *D* against a piece of soft rubber tubing, *E*, held in place by means of a clamp. The upper end of the tubing was closed by means of a glass plug. A pipette, *F*, was inserted in the lower end of the tubing. By turning the rod, it was thus possible to expel the air from the pipette and replace it with a solution containing the radioactive growth-regulator. A measured aliquot of a solution could then be forced out of the graduated pipette and applied by touching the tip of the pipette to a surface. When necessary, the tip of the pipette was used to spread the liquid evenly over the plant surface. A removable clamp, *G*, was used, so that the pipette could be easily detached and a new one inserted whenever desirable.

Cellophane disk method of plating ground samples of radioactive plant tissue

It is generally realized that the geometrical pattern of a sample is of importance in using end-window counters. Self-absorption must also be considered in preparing samples. It is possible to prepare samples of plant tissue having the same geometrical pattern and essentially one particle thick. The activity is then compared with that of a comparable layer from untreated plants upon which a known weight of the radioactive growth-regulator has been evenly distributed.

Grinding of material.—A medium-size Wiley mill was used in grinding the dried samples to 40-mesh dimension. The mill was not cleaned between samples representing replications of the same treatment. Between samples representing different treatments, as to either material applied or tissues sampled, however, the inside of the mill was washed with alcohol, and all parts were carefully cleaned with medicinal applicator swabs made of cotton and dipped into alcohol.

As a check on possible contamination from the mill a sample from untreated plants was occasionally ground, and its radioactivity determined. It was found that, by carefully washing the mill in the manner described, the radioactivity of ground samples from untreated plants was never significantly greater than the background, and generally a reading equal to the background was obtained. Thus, this method of cleaning the mill effectively prevented contamination of samples during the grinding process.

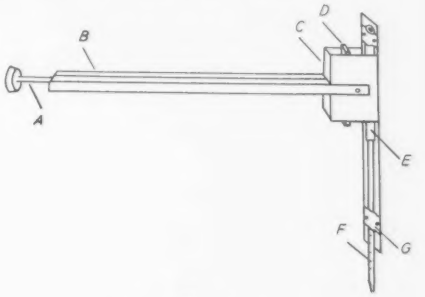

Fig. 1

Plating of sample.—In plating a sample of ground tissue, a brass ring having an inside diameter of 25 mm., an outside diameter of 31 mm., and a height of 3 mm. was pressed against the tacky surface of cellophane tape trimmed flush with the outside of the ring. Tape 1.5 inches in width was obtained for this purpose.[3] Thirty milligrams of ground tissue, thoroughly mixed, was placed on the tacky surface of the cellophane. The ring was held by forceps and gently tapped while it was revolved and tipped at an angle. In this way particles of tissue were rolled over the entire gummed surface inclosed by the ring. As particles adhered to the surface, others rolled over their

[3] Minnesota Mining and Mfg. Co., St. Paul, Minnesota.

tops, and finally the surface was evenly covered with a layer essentially one particle thick. The ring was then inverted and tapped, and the excess of tissue removed and weighed. The amount of tissue adhering to the cellophane was determined by subtracting the weight of the excess from the original amount used. An amount equal to that which adhered was weighed out and applied to additional disks to make duplicate plates. It was necessary to redetermine the weight of tissue that adhered to the cellophane disk whenever different kinds of plant parts were used, or when tissues from plants that received different treatments were encountered. Variation in the weight of plated samples that contained approximately the same number of particles was apparently due to differences in density of tissue in different parts of the plant or to differences in density that resulted from treatment.

The ring containing the sample on cellophane was then placed in a suitable holder, inclosed with a lead shield, and the radioactivity of the sample determined by means of an end-window counter. The activity of tissue plated in this manner was measured the same day that the disks were prepared, since it was found that occasionally the cellophane tended to buckle upon prolonged standing.

Comparison of radioactivity of tissue with known standard

It is sometimes desirable to express the counts obtained from a sample in terms of weight of plant growth-regulator in the sample. Following is a method of preparing a known standard sample and comparing it with an unknown one so that the activity of the latter can be expressed in terms of weight:

Tissues from untreated plants comparable with those of the treated ones were ground to 40-mesh dimension as previously described. Weighed amounts were then plated on cellophane disks. The amount of alcohol required to wet the tissue evenly was determined—0.06 ml. delivered dropwise.

A weighed amount of growth-regula-

TABLE 1

Data showing results obtained from typical experiment in which described methods were used to determine effects of various co-solvents and surface active agents on absorption of 2,4-dichloro-5-iodo^{131} phenoxyacetic acid by bean plants. Readings in millimicrograms

Treatment	Replications			Average
	1	2	3	
2,4-DI^{131} in water	137	178	216	177
" " " +Tween-20	875	812	700	796**
" " " +Emulphor-ELA	713	714	634	687**
" " " +Carbowax-1500	600	519	512	544**
" " " +Dreft	537	493	572	534**
" " " +Repcolene	482	450	460	464**
" " " +BRIJ-30	242	288	288	271*
" " " +Igepal	208	184	209	202
" " " +Nekal	178	194	178	183

** Significant at 1% level over that of the salt in water alone.
* Significant at 5% level over that of the salt in water alone.

tor was then dissolved in 95% ethyl alcohol, and dilutions were made to obtain concentrations equal to 0.25, 0.5, and 1.0 μgm. per 0.06 ml. aliquot, respectively. Tissue plated on three disks was then moistened with 0.06-ml. aliquots of the respective solutions, the alcohol was allowed to evaporate at room temperature, and the radioactivity of each sample was determined. A straight line was obtained when the counting rate was plotted against millimicrograms of radioactive growth-regulator used. The readings were corrected for decay when necessary. It was learned that the relationship between radioactivity and weight of the growth-regulator used for the standards was not a straight-line function except when the layer of tissue on the cellophane was essentially one particle thick.

Through the use of the above methods of treating plants, preparing samples, and measuring radioactivity, sufficient accuracy and reproducibility were obtained to give highly significant differences between treatments. Typical data obtained through the use of these methods are illustrated in table 1. It should be stressed that, in evaluating the usefulness of these methods, all sources of variation were considered, including that resulting from plant variation.

It should be mentioned also that difficulty was encountered in preventing the growth-regulating mixtures from spreading over the lanolin and onto tissue outside the inclosed area when these mixtures were applied under very humid conditions. This difficulty was not encountered when the mixtures were applied during a period of relatively low humidity.

The above methods were found to be useful for tracer studies with plants. It is quite probable that they could be altered so as to be useful in other types of experiments where tracers are used.

Summary

1. An inexpensive applicator made to hold radioactive solutions and mixtures in a pipette at a distance from the operator is described. As little as 0.01 ml. of solution can readily be delivered and placed on a plant with this apparatus.

2. A simple method of plating samples of ground radioactive plant tissues on the tacky surface of cellophane is described. The method makes possible the measurement of radioactivity in a weighed sample of ground tissue spread in an even layer essentially one particle in thickness.

3. Evaluation of counts in terms of weight of the tagged compounds was accomplished by impregnating a plated sample of tissue with a known amount of the tagged compound.

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EFFECT OF DDT ON GERMINATION OF CERTAIN SEEDS

H. T. HOPKINS and EBEN H. TOOLE

Introduction

From time to time statements have been made that DDT affects the germination of certain seeds. Experiments were set up to measure the magnitude of the effects, if any, using two kinds of DDT and four different types of seeds.

Material and methods

Special crepe paper, with the name "Kimpack," was placed in 8 × 8-in. waxed cardboard boxes and impregnated with 175 ml. of a 10% solution of technical DDT (m.p. *ca.* 90° C.) or p,p′-DDT (m.p. *ca.* 108° C.) in acetone. After a suitable time during which the acetone was allowed to evaporate, the papers were moistened with tap water. Fifty seeds of Henderson's bush lima bean (*Phaseolus lunatus*) or Early Prolific squash (*Cucurbita pepo*) or one hundred seeds each of Early Fortune cucumber (*Cucumis sativus*) or Abruzzi rye (*Secale cereale*) were placed in each box. Special paper towels, also impregnated with DDT, were moistened and placed on top of the seeds; nonimpregnated paper was used for the controls. The boxes were then placed in germinating chambers under conditions of controlled temperature and humidity, rye seeds at 20° C. and the others at 25° C. Sixteen replicates of lima bean, squash, and rye, and twelve of cucumber were employed.

Results

The mean percentage germination after 7 days (8 days for squash) is shown in table 1. The effect of DDT on early growth of certain seedlings is shown in table 2 and figures 1 and 2. The system of evaluation of sprouted seeds was that currently in use by seed technologists (2). Germination is defined as the number of seeds which swelled, sprouted, and met all the requirements of a normal seedling. Hard seeds of lima bean and sound seed of Abruzzi rye were excluded in calculating percentage germination.

TABLE 1

MEAN GERMINATION (PER CENT) AS AFFECTED BY DDT

Treatment	Lima bean*		Cucumber*	Rye*	Squash†
	Seed planted flat	Seed planted with hilum down	Seed planted flat	Seed planted flat	Seed planted flat
Technical DDT	98.3	84.2	93.7	78.2	71.1
p,p′-DDT	97.1	91.6	93.4	81.2	80.2
Control	99.1	96.2	94.1	81.6	85.5
$LSD_{5\%}$	2.7	3.2	1.8	2.8	5.8
$LSD_{1\%}$	3.7	4.3	2.5	3.7	7.9

* After 7 days. † After 8 days.

TABLE 2

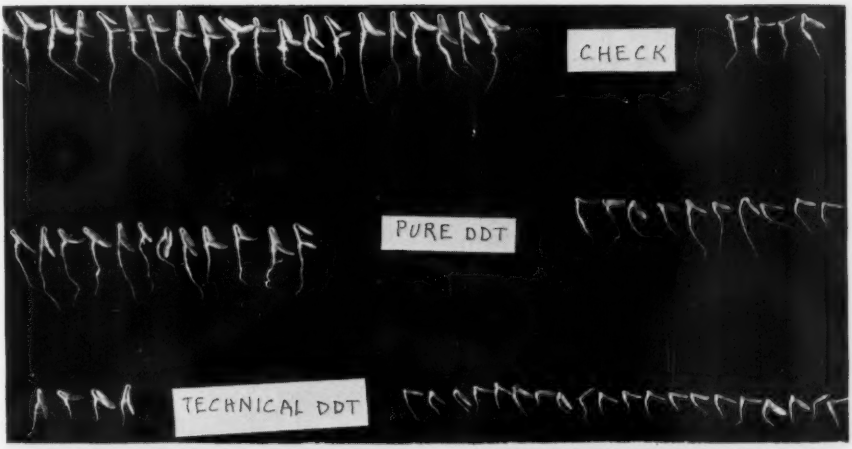
EFFECT OF DDT ON EARLY GROWTH OF SEEDLINGS, 7 DAYS FROM PLANTING

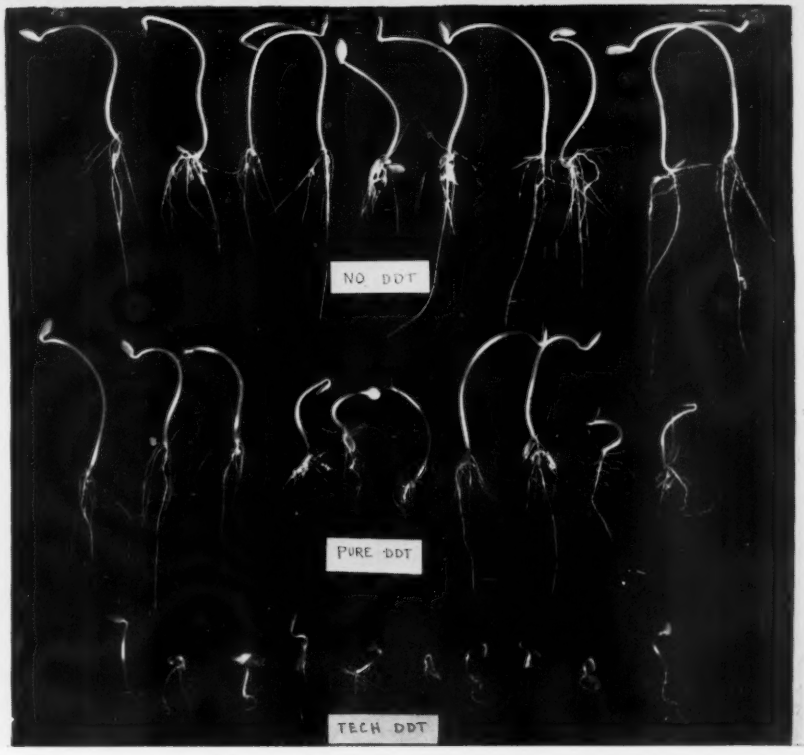
Treatment	Lima bean* (%)	Cucumber† (%)	Rye‡ (%)
Technical DDT	20.5	27.6	59.1
p,p′-DDT	50.1	51.4	72.9
Control	62.8	80.2	76.1
$LSD_{5\%}$	6.9	8.5	4.2
$LSD_{1\%}$	9.2	11.6	5.7

* Mean percentage of seedlings having hypocotyls which grew 3 cm. or more in length.

† Mean percentage of seedlings shedding seed coats.

‡ Mean percentage of seedlings having plumule at least three-fourths the length of sheath and having three roots.





FIGS. 1, 2.—Effect of DDT on early growth of seedlings. Fig. 1 (*above*), Early Fortune cucumber; criterion of effect was mean number of seedlings shedding seed coat 4 days from planting. Photograph shows approximately one-half of one replicate picked at random from each treatment; seedlings which have shed seed coats on left. Fig. 2 (*below*), Early Prolific squash, showing inhibition of root and hypocotyl elongation 7 days from planting. Photograph shows one-fifth of one replicate picked at random from each treatment.

The range in average number of such seeds fell between one and two per box.

Discussion

The data in table 1 indicate the essential lack of effect of p,p′-DDT on germination of four different types of seeds, except in the case of lima bean planted with hilum down. Technical DDT, however, significantly depressed germination of rye, squash, and lima-bean seeds, the latter only when planted with hilum down. Thus some component present in Technical DDT other than p,p′-DDT inhibited germination of these seeds at the high level of application used. Three lots of Technical DDT analyzed by HALLER *et al.* (1) were found to contain up to ten different compounds. Preliminary data indicate that o,p′-DDT and 2-trichloro-1-p-chlorophenylethanol are the most toxic compounds occurring in Technical DDT; the latter is inhibitory to germination at high concentrations. The marked effect of both grades of DDT on germination of Henderson bush lima-bean seed planted with hilum down was linked to the tendency of upwards of seven seeds per box to disintegrate before germination could be completed. The effect of p,p′-DDT on squash germination approached significance at the 5% point.

Early growth of lima-bean, cucumber, and rye seedlings was profoundly affected by DDT in the high concentration employed in this study (table 2; figs. 1, 2). Work is continuing on the effects, on growth, of DDT and other water-insoluble compounds which might accumulate in soils.

Summary

Pure DDT (p,p′-DDT) was shown to have no effect on the percentage germination of lima-bean, cucumber, rye, or squash seeds planted flat onto special crepe paper impregnated with a large amount of this compound. A significant effect of p,p′-DDT on germination of lima-bean seed was noted only when planted with the hilum down; this effect was characterized by a disintegration of the seed before germination could be completed. A depressing effect of Technical DDT is thought to be due to the presence of some component other than p,p′-DDT, which became inhibitory or toxic at the high level of application used in this work.

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ANATOMICAL EFFECTS OF LYGUS INJURY TO GUAYULE

F. T. ADDICOTT[1] and V. E. ROMNEY[2]

Lygus bug injury has been reported in over fifty species of plants of economic value. Descriptions of the effects of their feeding on alfalfa have been given by SHULL *et al.* (3), who showed that *Lygus hesperus* Kgt. caused a reduction in the growth of alfalfa plants, and by CARLSON (1), who showed that disintegration of tissues occurred adjacent to *Lygus* feeding punctures in young alfalfa seed pods. In guayule (*Parthenium argentatum*) ROMNEY *et al.* (2) showed that growth was greatly retarded by the feeding of *L. hesperus*. They observed the insect ovipositing in the flower heads and both nymphs and adults feeding on the developing seed. When seed was absent or became depleted, the insect fed on the growing stem tips, causing local cessation of meristematic activity and growth. Four or five weeks after removal of the insects, growth was resumed by the development of lateral buds 10 mm. or so below the injured stem tip. A much higher mortality of the insects occurred when they fed on the stem tips than when they fed on the seed.

In an attempt to determine the extent of the injury caused by the *Lygus* bugs, six young vegetative plants of guayule and twenty-five adults of *L. hesperus* were placed in a cage in a greenhouse beside an insect-free cage containing six sister plants. Ten days later the infested plants showed considerable injury of their branch tips. All the leaves up to 2–3 cm. in length were gray and withered. The youngest parts of the branch tip were the most seriously affected, with stem apex and surrounding young leaves severely shrunken.

Samples of stem tissue were taken at this time, fixed, sectioned, and stained with safranin and hematoxylin. Comparable sections of infested and uninfested plants are shown in figures 1–6.

Tissues at the stem apex are shown in figures 1 and 2: 1 from an uninfested, and 2 from an infested plant. In the latter the cells are collapsed and the details of their structure obliterated. Figures 3 and 4 show tissues behind the stem apex: 3 from an uninfested, and 4 from an infested plant. Damage here is less extensive; areas of drastically injured, often collapsed cells, are interspersed with areas of intact cells. Tissues 10 mm. from the stem apex of infested plants are shown in figures 5 and 6. Damage here, largely in the phloem region, is much less than in the regions distal to it. The evacuated egg sac shown in figure 5 and the developing embryo in figure 6 indicate that numerous *Lygus* eggs are deposited in this region.

These observations support the idea that *Lygus* in its feeding injects a toxic material into the host. The cessation of longitudinal growth after a few days of *Lygus* feeding is the result of the death of the terminal meristem, the young leaves, and much of the phloem near the stem tip. That the *Lygus* toxin is not highly virulent is shown by the restriction of the injuries to the area of feeding and by the eventual development and growth of lateral buds close to the stem tip.

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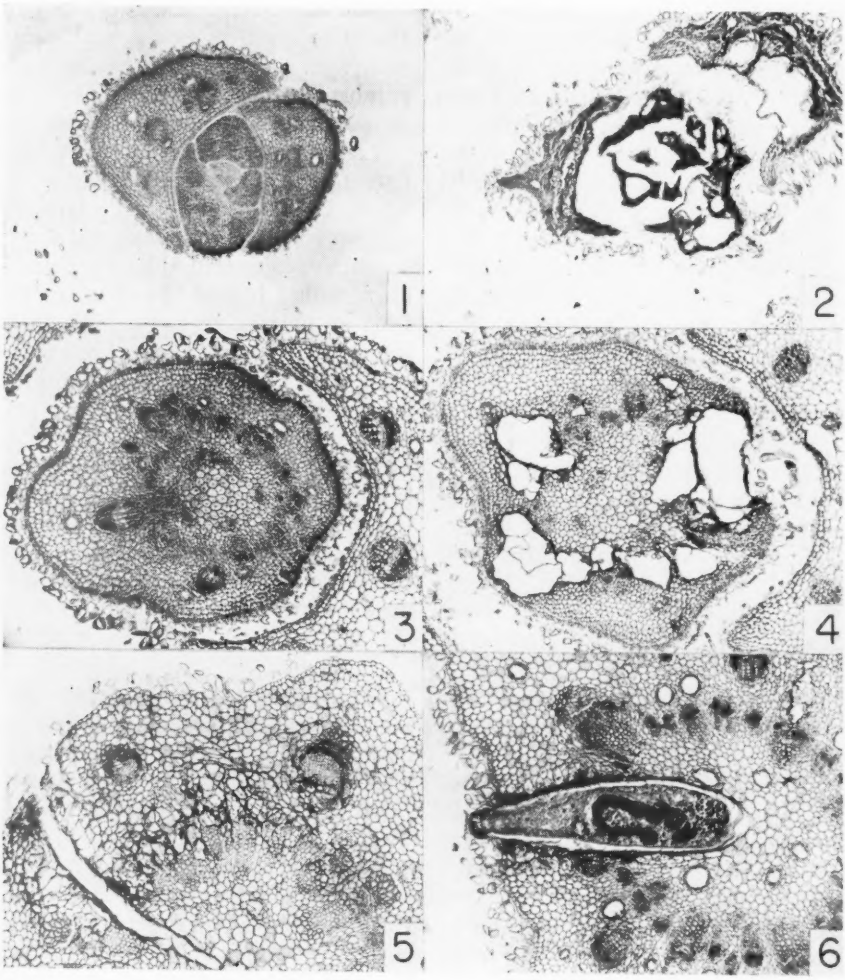

FIGS. 1–6.—Cross sections of stem of guayule, uninjured or injured by *Lygus hesperus*. ×45. Figs. 1, 2, at stem apex; most central portion of tissue is stem tip; fig. 1, uninjured; fig. 2, injured. Figs. 3, 4, a few mm. behind apex; fig. 3, uninjured; fig. 4, injured. Figs. 5, 6, about 10 mm. behind apex, showing effects of injury, especially in region of phloem.

SELECTIVE INHIBITION OF THE GROWTH OF GREEN PLANTS AND FUNGI BY BETA METHYL UMBELLIFERONE[1]

Beta methyl umbelliferone has been found effective in inhibiting the growth of certain green plants and fungi. This compound is a substituted coumarin and undergoes many of the reactions of this class of compound. Coumarin has been reported by AUDUS and QUASTEL to be a selective phytocidal agent (1). A related compound umbelliferone occurs naturally in the bark of *Daphne mezereum* and in the resins galbanum and asafetida. The relation between the naturally occurring compounds and the synthetic product[2] suggests that the natural product may have antibiotic properties and that it may also inhibit growth of plants and fungi.

Seeds of Red Kidney bean, Alaska pea, Marketer cucumber, North Star corn, and White Banner wheat were planted in 4-inch unglazed clay pots in soil prepared by mixing equal amounts of sand, soil, and peat moss. Various amounts of beta methyl umbelliferone were added to the soil to adjust the concentrations in the entire soil mass to 500, 2000, and 8000 p.p.m.

The germination and growth of Red Kidney bean and cucumber seeds and seedlings were markedly inhibited, the degree of inhibition being in proportion to the concentration of the chemical (table 1, figs. 1, 2). The important point is that no formative effects or abnormal growth responses occurred other than the greatly reduced growth. Examination of the plants indicated that the inhibition was the result of suppressed root growth. The growth of wheat, on the other hand, was not affected by any of the concentrations used (table 1), while the growth of peas and corn was only slightly inhibited by a concentration of 8000 p.p.m. beta methyl umbelliferone.

Ultraviolet light acts to dwarf plants and apparently interferes with either the manufacture or the movement of natural growth hormones. It is suggested that beta methyl umbelliferone may inhibit plant growth because it acts to absorb in the plant large quantities of ultraviolet light, thus interfering with the normal functioning of the growth hormones.

Besides its action on green plants, beta methyl umbelliferone has also been found to inhibit the growth of three pathogenic fungi against which it has been tried. Replicated experiments were run, using *Poria monticola*, a wood-rotting fungus; *Fusarium lycopersici*, cause of tomato wilt; and *Monolinia fructicola*, cause of brown rot of stone fruits. The fungi were grown in Petri dishes containing 2% malt-extract agar to which were added various concentrations of umbelliferone. Table 2 shows the

TABLE 1

SELECTIVE INHIBITORY ACTION OF BETA METHYL UMBELLIFERONE, AS SHOWN BY FRESH WEIGHTS OF PLANTS HARVESTED 21 DAYS AFTER PLANTING IN CONCENTRATIONS NOTED

CONCENTRATION (P.P.M.) OF BETA METHYL UMBELLIFERONE IN SOIL	NO. OF PLANTS	WT. OF PLANTS (GM.)	WT. OF ROOTS (GM.)
	Cucumber		
Control	100	161.0	51.0
500	100	57.0	14.0
2000	100	47.5	11.0
8000	100	35.0	6.0
	Bean		
Control	100	715.0	260.0
500	100	600.0	195.0
2000	100	300.5	165.0
8000	100	200.0	25.0
	Wheat		
Control	100	109.2	52.0
500	100	106.5	47.6
2000	100	108.3	51.7
8000	100	104.8	48.7

[1] Journal article No. 1164 of the Michigan Agricultural Experiment Station.

[2] Synthesized and marketed by Koppers Chemical Co., Pittsburgh, Pa.

FIGS. 1, 2.—Effect of beta methyl umbelliferone in soil on growth. Fig. 1 (*below*), Red Kidney bean; *left*, grown in soil containing 2000 p.p.m. of chemical; *right*, control. Fig. 2 (*above*), cucumber plants growing in soil containing (*left to right*) 0, 500, 2000, and 8000 p.p.m. of beta methyl umbelliferone.

TABLE 2

EFFECT OF BETA METHYL UMBELLIFERONE ON GROWTH OF CERTAIN FUNGI

CONCENTRATION (P.P.M.) OF BETA METHYL UMBELLIFERONE	DAYS OF GROWTH					
	2	3	4	5	6	7
	Poria monticola					
20,000	6.5*	6.7	11.0	14.5	22.7	31.2
5,000	9.7	12.7	18.5	22.2	27.7	34.2
Control	17.0	29.0	41.7	56.0	70.0	83.0
	Fusarium lycopersici					
20,000	12.7	18.0	21.5	25.2	29.2	34.0
5,000	13.0	20.6	24.6	30.3	35.6	41.6
Control	22.0	33.3	44.0	53.7	64.0	73.7
	Monolinia fructicola					
20,000	9.0	15.0	18.2	22.5	28.7	31.5
5,000	12.2	17.2	22.0	27.7	34.0	38.2
Control	17.7	27.0	36.2	44.0	53.2	60.0

* Each figure represents average of diameter in millimeters of growth on four plates at 30° C.

average daily growth over a period of 7 days. The marked inhibition of these fungi by umbelliferone is particularly interesting because the compound is relatively soluble in water. This is a desirable characteristic for a fungus toxicant, since it means resistance to weathering.—C. L. HAMNER, H. M. SELL, WM. KLOMPARENS, and J. R. VAUGHN, *Departments of Horticulture, Agricultural Chemistry, and Botany and Plant Pathology, Michigan State College, East Lansing, Michigan.*

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A CONVENIENT METHOD OF GROWING FRUITING-BODIES OF A BRACKET FUNGUS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 616

In the course of some genetic work on *Schizophyllum*, difficulty was encountered in obtaining fruiting-bodies with regularity. The following method was therefore worked out and has proved entirely satisfactory.

Six-inch test tubes are used to contain the nutrient medium absorbed in a porous, air-containing filler. Each tube is first filled with vermiculite; a 5% malt-extract solution is then run slowly down the side of the tube to saturate the vermiculite. The composition of the nutrient solution does not seem to be important, since solutions of various composition have been substituted for the malt extract with equally good results. The tubes are covered with glass caps about ½ inch long and sterilized in the autoclave.

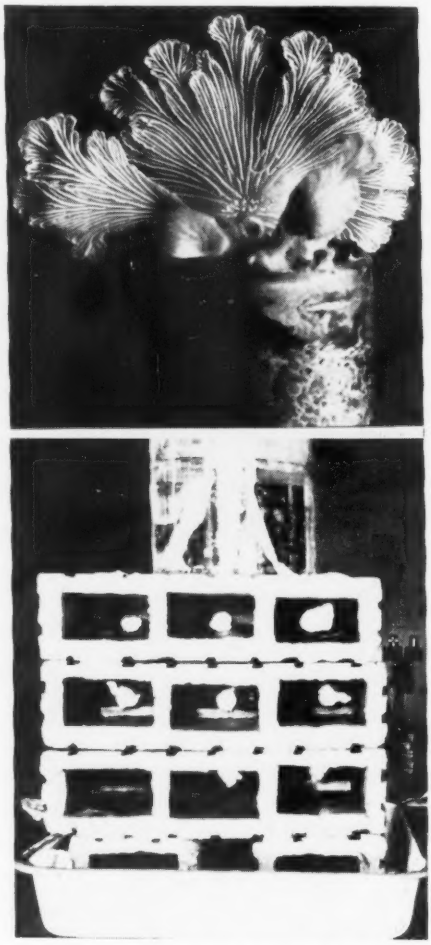
Each tube is then inoculated by placing a small cube of agar, taken from a Petri dish culture of the appropriate mycelium, on top of the vermiculite. They are incubated at room temperature in a vertical position, with their caps on, for about a week, or until a good growth of mycelium has covered the surface of the vermiculite and has grown about ½ inch down the tube. They are then ready for fruiting.

The fruiting-bodies are induced inside porous, hollow, soft tiles. Three or four such tiles, which can be obtained with three large holes and each with a size of 12″ × 12″ × 4″, are placed one on top of the other, with the holes running horizontally, in a suitable watertight tray (fig. 1). The uncapped test tubes are placed in the holes in an inclined position, with their open ends on a copper support and facing a window or other source of light.

In order to keep the tiles moist, a 3-liter jar of water is placed on top of them with two wicks leading from the jar to the top tile. In addition, two pieces of gauze hung over the open ends of the tiles reach down into the tray of water. After an initial soaking of the tiles and gauze their moist condition can be maintained by filling the jar every day or second day; water is emptied from the tray when necessary.

Growth of molds or bacteria on the tiles can be prevented by dissolving a few grams of copper sulfate in the water.

Within 1–5 weeks after the test tubes have been placed in the tiles two or three fruiting-bodies will be found growing out of the end of each tube (fig. 2).—H. P. PAPAZIAN, *Department of Botany, University of Chicago.*



FIGS. 1, 2.—Fig. 1 (*below*), tiles with front gauze removed to show fruiting-bodies. Fig. 2 (*above*), mature fruiting-body projecting from end of test tube.

A METHOD OF ISOLATING THE FOUR SPORES FROM A SINGLE BASIDIUM IN SCHIZOPHYLLUM COMMUNE

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 617

Tetrad analysis, in which the four products of a meiotic division are separately analyzed, has been used in genetic studies on a variety of organisms during the last 25 years. It is only very recently (**3, 5**) however, that the deductions that can be drawn from such data have been adequately investigated and that the full advantages of the method have become apparent.

A new technique for isolating the tetrad of spores from a basidium is described below. The method was developed in order to make tetrad analyses in the bracket fungus, *Schizophyllum*. This organism offers many advantages over other basidiomycetes as a laboratory subject but has the disadvantage that the methods of obtaining tetrads hitherto described (**1, 2, 4**) are not applicable.

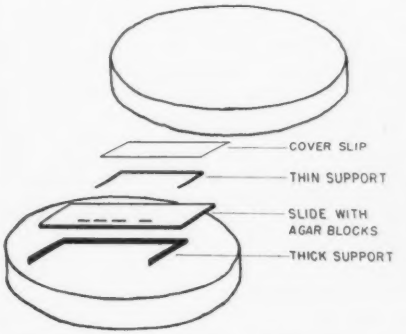


FIG. 1.—Exploded diagram of moist chamber

The method consists essentially of cutting sections of the lamellae and allowing basidia to develop on the hymenial surface of these while they rest on agar blocks in a moist chamber. The spores are then detached with the use of a microdissector and isolated in two stages, since the spores are so small that they can only be conveniently handled, and finally isolated, after germination.

The moist chamber (fig. 1) consists of a Petri dish containing 1 or 2 ml. of water in which is placed a microscope slide resting on a thick support and on this slide a long cover slip which, in turn, rests on a thin support. The thick and the thin supports can be made from 12-mm. and 8-mm. glass rods, respectively, bent as illustrated. Several such moist chambers should be prepared and sterilized in the autoclave together with a short section of pith, after which several rectangular blocks of 4% agar about 1 cm. × 3 mm. and 3 mm. thick are placed in line on the slide about 5 mm. from the edge. They should be spaced so as to leave room for an additional similar agar block to be introduced at a later stage. It is important that the thickness of the agar blocks should be as nearly equal as possible and that there is a space of at least 4 mm. between the top of the agar blocks and the cover slip above them.

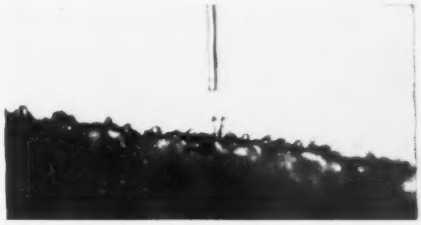


FIG. 2.—Photomicrograph of hymenial surface with glass needle opposite a tetrad of basidiospores.

The sections of lamellae can then be introduced. For this purpose either fresh fruiting-bodies, or dried ones that have been well soaked in distilled water, can be used. Single lamellae are cut out of the fruit and sectioned freehand, about 50 μ thick, in the sterilized pith, sections being cut along the long axis of the lamella. These sections are then laid one on each of the agar blocks contained in the moist chambers in such a way that their hymenial surfaces lie at right angles to the top surface of the block and one surface faces outward under the cover glass.

After about 5 hours at room temperature numerous basidia with basidiospores will be seen laterally along the sections. An additional block of agar is then placed in line with the others. A tetrad of spores is then removed by a fine glass needle mounted in a microdissector (fig. 2) and

deposited on the additional, bare block, where the four spores are spaced at intervals along the length of the block. An 8-mm. objective has been found most suitable for this operation, and the glass needle may, with advantage, be dipped in hot olive oil before use. The additional block is then removed to a sterile van Tieghem cell, a new block replaced, and the operation repeated.

After about 15 hours at room temperature the young mycelia are plainly visible, under a dissecting microscope, on the agar blocks in the van Tieghem cells. Each block can then be cut into four smaller blocks, each containing one plant, and transferred to separate Petri dishes for subsequent studies.—H. P. PAPAZIAN, *Department of Botany, University of Chicago.*

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CURRENT LITERATURE

Genetics, Paleontology, and Evolution. Edited by G. L. JEPSEN, G. G. SIMPSON, and ERNST MAYR. Princeton: Princeton University Press, 1949. Pp. xiv+474. $6.00.

Publication of this volume upon various aspects and implications of organic evolution signifies the formal completion of a task by the Committee on Common Problems of Genetics, Paleontology, and Systematics. This committee was established on February 6, 1943, as a joint or interdivisional committee by the Division of Geology and Geography and the Division of Biology and Agriculture of the National Research Council.

In 1946 Princeton University extended an invitation to the committee to hold the final symposium under the sponsorship of the university in connection with its Bicentennial Conferences. As a consequence of this invitation an international Conference on Genetics, Paleontology, and Evolution was held at the Princeton Inn on January 2–4, 1947, and the papers presented there constitute the present volume.

Following a Foreword the book is divided into eight sections. Section I, "Geological Time," contains one paper, "Time in Earth History," by ADOLPH KNOPF; Section II, "Viewpoints on Evolution," contains five papers: "Gene and Character," by CURT STERN; "Gene Homologies and Mutants of *Drosophila hydei*," by WARREN P. SPENCER; "The Evidence Afforded by Fossil Vertebrates on the Nature of Evolution," by D. M. S. WATSON; "Comparative Anatomy and the Evolution of Vertebrates," by D. DWIGHT DAVIS; and "Some Aspects of Plant Morphology and Evolution," by THEODOR JUST; Section III, "Evolutionary Trends," contains four papers: "Time Series and Trends in Animal Evolution," by ALFRED S. ROMER; "On the Evolution of the Dipnoi," by T. STANLEY WESTALL; "Evolutionary Rates and Trends in Rhinoceroses," by HORACE E. WOODS, II; and "Evolutionary Trends in the Angiosperms," by RALPH W. CHANEY; Section IV, "Evolutionary Rates," contains three papers: "Rates of Evolution in Animals," by GEORGE G. SIMPSON; "Rates of Evolution in Plants," by G. LEDYARD STEBBINS, JR.; and "Rates of Evolution in Taeniodonts," by BRYAN PATTERSON; Section V, "Speciation," comprises six papers: "Speciation and Systematics," by ERNST MAYR; "The Significance of Ecological Isolation," by DAVID LACK; "Early Stages in Allopatric Speciation," by E. B. FORD; "Patterns of Evolution in the Genus *Rana*," by JOHN A. MOORE; "Increased Variability in Populations Following Natural Hybridization," by WILLIAM HOVANITZ; and "Evidence for the Genetic Submergence of *Pinus remorta*," by HERBERT L. MASON; Section VI, "Adaptation," consists of two papers: "Adaptation and Selection," by SEWELL WRIGHT, and "Progressive Adaptations as Seen in the Fossil Record," by EDWIN H. COLBERT; Section VII, "Human Evolution," one paper: "Human Evolution: Past and Future," by J. B. S. HALDANE; and Section VIII, "Summation, Redintegration of the Symposium on Genetics, Paleontology, and Evolution," by H. J. MULLER. Finally, there is an adequate glossary and an index.

There is brought together in this single volume a large and diverse mass of material bearing on the three topics; and, although a synthesis of viewpoints has not been attained, yet it can be said that definite progress has been made. There is reason to hope that additional conferences of the leaders in these fields may serve to bring further progress toward this goal.—J. M. BEAL.

The Whole Life Cycle of Chromosomes and Their Coiling Systems. By L. R. CLEVELAND. "Transactions of the American Philosophical Society, New Series," Vol. **39**, Part 1. Philadelphia: American Philosophical Society, 1949. $1.50.

This paper of some 100 pages, containing 35 excellent plates, is one of the significant cytological contributions of recent years. It presents the results of critically detailed studies of the chromosomes of seven families and forty genera of hypermastigote flagellates with the object "not only to clear up certain of the unknown events in the life cycle of the chromosomes but also to point out some of the erroneous conclusions that have been made regarding the manner in which relational coiling is produced and disappears, the relation of centromere to chromosome, relic coiling, matrix, and chromonema." It also aims "to give such an accurate and complete account of all the events in the life cycle of chromosomes and their coiling systems that it will dispel some of the vague generalities and other speculations that have been tossed about for many years without having advanced our knowledge of cytology very much if at all."

The flagellates employed in these investigations possess two or four large chromosomes in which the major, minor, and supercoils, as well as relational coiling and chromosomal twisting, can be seen remarkably clearly. Chromosome structure is distinctly visible at all stages in mitosis, and it is possible to follow all the changes which the chromosomes undergo through an entire mitotic cycle. It is demonstrated that minor coils exist throughout the life-cycle of a chromosome during a mitotic cycle. Chromosomes of two types are presented, those with single and those with double chromatids. The centro-

mere is not merely a part of, or a point on, a chromosome but something which leads an independent existence. It plays a more important role in nuclear division than has been previously assigned to it. In many of the chromosomes used as illustrations the centromere is definitely terminal in position, while in others it may be median or submedian.

This contribution is strongly recommended to all cytologists and to biologists in general. It is the earnest hope of the reviewer that a similar study of meiosis may soon be presented by Dr. CLEVELAND.—J. M. BEAL.

Traité de cryptogamie. By LOUIS LUTZ. Paris: Masson & Cie, 1948. Pp. 708 + 4 color pls. Fr. 1450.

This book is a text for a course on cryptogams given to pharmacy students, and the subject matter is selected accordingly. More than three-fourths of it is devoted to the fungi, and the rest covers the algae, bryophytes, and pteridophytes. The biochemical aspect is emphasized throughout, particularly the chemistry of fungal pigments, but there are some notable omissions. Thus, no mention is made of the biochemical mutants in *Neurospora*, and, although a page is devoted to sex hormones in mammals, there is no reference to sex hormones among the thallophytes. There is a long description of the incompatibility factors in the basidiomycetes, but, unfortunately, the details are not quite accurate, and the reader is hampered, as elsewhere in the book, by the lack of references.

The book will be of greatest value to those scientific gastronomes who are interested in the details of such varied topics as cheese- and liquor-making, the culinary merits of various truffles, the biology of the green oyster, and in the physiology of mushroom poisoning.—HAIG PAPAZIAN.

Woody-Plant Seed Manual. Prepared by the FOREST SERVICE. U.S. Dept. Agr. Misc. Pub. 654. Washington: Government Printing Office, 1948. Pp. vi + 416. Illus. $2.75.

Although written primarily for the practical grower, this work constitutes a valuable sourcebook for the research worker interested in seed physiology, especially the extensive information it may provide about research material. The first of the two parts contains general information about seeds, their nature, and their handling; such topics as seed structure and types, the germination process, dormancy, storage, viability problems, and methods of seed testing are given comprehensive treatment. Consideration is made of factors in seed production and dispersal, and the importance of seed source is stressed. The commercial problems of extensive collection and extraction of seeds are well treated.

In Part II, generic and specific treatment is made of 444 species; genera are arranged in alphabetical order. Under each genus or species the distribution, use, seeding habits, collection and storage, germination, and methods of nursery and field practice are considered. This section is wellillustrated with drawings or photographs of intact seeds and seed sections showing not only general appearance but relative size and position of the embryo. Line drawings illustrating early seedling habits are frequent. Many tables include synonyms of scientific names, plant form, range, use, and other general information. In many cases, specific presowing treatments and conditions for germination are summarized in tabular form. For relatively large genera, the purity, soundness of seed, number of seeds per pound, and cost per pound are given for the important species.

At the end of each part, a bibliography is included, that dealing with Part II being arranged alphabetically by genus.

The manual also includes a use list of species which is divided into sections separating those employed in wood production, erosion control, shelter belts, wildlife purposes, and ornamental planting. A brief glossary and an index of plant names used in Part II are provided.

In addition to the information from over four hundred books and publications, much use has been made of unpublished work. Within the limits of the reviewer's knowledge, the drawings of seedlings, information on germination conditions, and methods given for the breaking of dormancy are correct.

Such a comprehensive work as this has long been needed. In terms of both organization and information provided there is no question about the utility of this compilation for anyone working with seeds of woody plants.—S. M. SIEGEL.